NATRIURETIC PEPTIDE RECEPTORS IN THE ATLANTIC HAGFISH, MYXINE GLUTINOSA

BY

MARIE-THERESE TOOP

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1994

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr David H. Evans, who offered a place and support for the completion of this doctoral study, and who, together with the other members of my committee, Drs Michele Wheatly, Lou Guillette, Mike Miyamoto, and Debopam Chakrabarti, gave generously of time and energy. This research was supported by National Science Foundation Grant DCB 8916413 to David Evans and by NIH EHS-P30-ESO3828 to the Center for Membrane Toxicity Studies at Mount Desert Island Biological Laboratory in Maine. I thank Dr Frank Nordlie for being present at my defense in the absence of Dr Mike Miyamoto, and also, as Chair, for his consideration during my studentship in the Zoology Department. The use of Dr Larry McEdward's microscopes, cameras, and video equipment is greatly appreciated. Dr Frank J. Maturo Jr. is thanked for his friendship and advice, and especially for quoting a stanza from Thomas Gray's 'Elegy in a Country Churchyard' at a critical time in my career. Dr John Donald's assistance has been invaluable throughout every phase of this dissertation. It is impossible to imagine its completion without his friendship and support. Several past and present graduate students and friends must be acknowledged for both the tangible and the intangible: Kent Vliet, Vince DeMarco, John Payne, Richard Buchholz, Evan Chipouras, Andy Rooney, Daryl Harrison, Simon Sellers, Irene Poyer, Helen Madaras, and Ruth Lederman. I thank my family -- as always; they never imagined I would be quite so extreme in taking my father's advice that 'the best thing a woman can do is get a decent education'. Finally, I especially thank my son, Jake Sellers, who will (I hope), when he is quite grown, recall the times spent in the company of hagfish and his mother .

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ii

LIST OF FIGURES v

ABSTRACT vii

GENERAL INTRODUCTION 1
Hagfish and the Environment of the Early Vertebrates 1
Natriuretic Peptides and their Receptors 3
Natriuretic Peptides and Osmoregulation in Fishes 6
Fish Osmoregulation 6
Natriuretic Peptides in Fishes 7

NATRIURETIC PEPTIDE RECEPTORS IN THE GILLS OF THE ATLANTIC HAGFISH, MYXINE GLUTINOSA 11
Introduction 11
Hagfish Gill Morphology 11
Natriuretic Peptides and Fish Gills 12
Materials and Methods 15
Animal Maintenance 15
Autoradiography 15
Membrane Preparation 16
Radioligand Binding Assays 17
Affinity Cross-Linking 17
Guanylate Cyclase Assays 18
Data Analysis 18
Results 19
Autoradiography 19
Radioligand Binding Assays 19
Saturation binding 19
Competition Binding 20
Affinity Cross-Linking and SDS-PAGE 20
Guanylate Cyclase Assays 21
Discussion 21

NATRIURETIC PEPTIDE RECEPTORS IN THE DORSAL AND VENTRAL AORTAE, AND THE KIDNEYS OF THE ATLANTIC HAGFISH, MYXINE GLUTINOSA 37
Introduction 37
Natriuretic Peptides and the Mammalian Kidney 37
Natriuretic Peptides and the Fish Kidney 40
Materials and Methods 43
Autoradiography 43
Competition Binding Assays 44
Guanylate Cyclase Assays 45

Results 45
Autoradiography 45
Competition Binding Assays 46
Guanylate Cyclase Assays 46
Discussion 47

WHOLE ANIMAL VOLUME REGULATION, AND NATRIURETIC PEPTIDE RECEPTORS, IN THE ATLANTIC HAGFISH, MYXINE GLUTINOSA, EXPOSED TO 85 % AND 115 % SEA WATER 61
Introduction 61
Volume Regulation in Hagfish 61
Natriuretic Peptides and Volume and Salt Loading in Mammals 62
Natriuretic Peptides and the Environmental Salinity of Fish 63
Materials and Methods 65
Volume Regulation and Tissue Preparation 65
Competition Binding Assays 66
Results 66
Volume Regulation, Plasma Osmolality, and Hematocrits 66
Competition Binding Studies 67
Gill membranes 67
Kidney glomerular sections 68
Discussion 68

GENERAL DISCUSSION 80

LIST OF REFERENCES 88

BIOGRAPHICAL SKETCH 99

LIST OF FIGURES

Figure 2-1. Diagram of the position of gills in the hagfish body, and longitudinal section through a single gill, indicating major gill regions.....26

Figure 2-2. Autoradiographs of four serial longitudinal sections of a single hagfish gill pouch incubated with ^{125}I-ANP alone (A) or in the presence of various unlabelled NPs (C-D).....27

Figure 2-3. Autoradiographs of four serial longitudinal sections of a single hagfish gill pouch incubated with ^{125}I-CNP alone (A) or in the presence of various unlabelled NPs (C-D).....28

Figure 2-4. Light micrograph of longitudinal gill sections dipped in X-ray sensitive emulsion showing the distribution of specific binding in the lamellar region.....29

Figure 2-5. Saturation analysis of ^{125}I-ANP specific binding to gill membranes. 30

Figure 2-6. Saturation analysis of ^{125}I-CNP specific binding to gill membranes. 31

Figure 2-7. Competition study indicating the relative abilities of rANP, pCNP, and C-ANF at increasing concentrations to compete for ^{125}I-ANP specific binding sites.....32

Figure 2-8. Competition study showing the relative abilities of rANP, pCNP, and C-ANF at increasing concentrations to compete for ^{125}I-CNP specific binding sites.....33

Figure 2-9. Autoradiograph of SDS-PAGE of hagfish gill NP binding sites affinity cross-linked with iodinated NPs under reducing conditions.....34

Figure 2-10. Effects of natriuretic peptides on cGMP production rate in hagfish gill membrane preparations.....35

Figure 2-11. Model of NP receptors in hagfish gills.....36

Figure 3-1. Diagram of the position of kidneys and dorsal aorta in the hagfish trunk, indicating major structures.....51

Figure 3-2. Autoradiographs of ^{125}I-ANP binding in serial longitudinal sections through both hagfish kidneys and dorsal aorta.....52

Figure 3-3. Autoradiographs of ^{125}I-ANP binding in serial longitudinal sections through the hagfish ventral aorta.....53

Figure 3-4. Autoradiographs ^{125}I-CNP binding in longitudinal sections through both hagfish kidneys and dorsal aorta.....54

Figure 3-5. Enlargement of autoradiograph of ^{125}I-ANP binding in longitudinal sections through both hagfish kidneys and dorsal aorta, showing specific regions of tissue.....55

Figure 3-6. Light micrograph of longitudinal sections of glomeruli (A,B) and dorsal aorta (C,D) dipped in X-ray sensitive emulsion showing the distribution of ^{125}I-ANP specific binding.....56

Figure 3-7. Light micrograph of longitudinal sections of neck segment (A,B) and archinephric duct (C,D) of the hagfish kidney dipped in X-ray sensitive emulsion showing the distribution of ^{125}I-ANP specific binding.....57

Figure 3-8. Autoradiographs of longitudinal hagfish kidney sections showing displacement of ^{125}I-ANP specific binding at various concentrations of rANP.....58

Figure 3-9. Competition study indicating the relative abilities of rANP, pCNP, and C-ANF at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish glomeruli.....59

Figure 3-10. Effects of natriuretic peptides on cGMP production rate in hagfish kidney membrane preparations.....60

Figure 4-1. Histogram of % weight changes in hagfish exposed to 85 %, 100 %, and 115 % SW during a 15 day period.....73

Figure 4-2. Competition study indicating the ability of rANP at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish gills exposed to 85 %, 100 %, and 115 % SW.....74

Figure 4-3. Competition study indicating the ability of pCNP at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish gills exposed to 85 %, 100 %, and 115 % SW.....75

Figure 4-4. Competition study indicating the ability of C-ANF at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish gills exposed to 85 %, 100 %, and 115 % SW.....76

Figure 4-5. Competition study indicating the ability of rANP at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish glomeruli exposed to 85 %, 100 %, and 115 % SW.....77

Figure 4-6. Competition study indicating the ability of pCNP at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish glomeruli exposed to 85 %, 100 %, and 115 % SW.....78

Figure 4-7. Competition study indicating the ability of C-ANF at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish glomeruli exposed to 85 %, 100 %, and 115 % SW.....79

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

NATRIURETIC PEPTIDE RECEPTORS IN THE ATLANTIC HAGFISH, MYXINE GLUTINOSA

By

Marie-Therese Toop

August, 1994

Chairman: Professor David H. Evans
Major Department: Zoology

The Atlantic hagfish, Myxine glutinosa, is a marine osmoconformer that diverged from other vertebrates over 500 million years ago. Natriuretic peptides (NPs) are involved in salt and water homeostasis in mammals and are implicated in fish osmoregulation. Natriuretic peptide receptors (NPRs) in the gills, kidney, and aortae of the hagfish were examined in normal and salinity adjusted animals, using tissue section autoradiography, radioligand binding assays, affinity cross-linking, SDS-polyacrylamide gel electrophoresis, and guanylate cyclase (GC) assays. Two NPRs were found in the gill: the first (site 1) preferentially bound atrial natriuretic peptide (ANP, Kd = 15 pM; B_{max} = 50 fmol/mg protein), and to a lesser extent C-type natriuretic peptide (CNP, Kd = 380 pM; B_{max} = 120 fmol/mg protein); the second (site 2) bound both ANP and CNP with similar affinities (Kds: 15 pM and 13 pM respectively) but the B_{max} for CNP was lower (23 fmol/mg protein as opposed to 50 fmol/mg protein for ANP). ANP, CNP, and C-ANF (a specific ligand of the mammalian clearance receptor, NPR-C) competed for ^{125}I-ANP and ^{125}I-

CNP binding sites. The apparent molecular mass of both hagfish NPRs was 150 kDa; no 65-75 kDa receptor, indicative of NPR-C, was observed. The kidney, and the dorsal and ventral aortae contained a single population of site 1 receptors, since C-ANF and CNP failed to compete for ^{125}I-ANP binding sites, and ^{125}I-CNP did not bind. ANP and CNP stimulated cGMP production above basal levels in the gills and kidney, but C-ANF did not, suggesting that site 1 is GC-linked. Hagfish in 85 % sea water successfully volume regulated, but those in 115 % sea water did not. Hagfish NPRs in high and low salinities showed increased sensitivity to CNP. This study indicates that GC receptors are an ancient vertebrate characteristic; that the principal receptor type (site 1) is similar to the ANP receptor of mammals; and that the gill site 2 receptor is not structurally homologous to the mammalian NPR-C. It is concluded, therefore, that the vertebrate NP system was already elaborate before the hagfish diverged from the other vertebrates.

GENERAL INTRODUCTION

Hagfish and the Environment of the Early Vertebrates

Hagfish (Superclass Agnatha, Class Myxini, Order Myxiniformes, Family Myxinidae) hold a pivotal and controversial position in the history of vertebrates, hinging on whether vertebrates arose approximately 500 million years ago (MYA) in a marine or freshwater environment; a question that is still debated today (Griffith, 1985; Halstead, 1985). Hagfish are the only marine osmoconforming craniates, maintaining their blood at a concentration almost indistinguishable from sea water, and sharing osmotic strategies with marine osmoconforming invertebrates (Robertson, 1957; Bellamy and Chester Jones, 1961; Robertson, 1963; Cholette et al., 1970; Hardisty, 1979). The most parsimonious hypothesis, given the similarity between the myxinoid and the marine invertebrate osmotic profile, is that hagfish evolved in sea water and therefore did not secondarily derive osmoconformity. The arguments for a freshwater, or at least a brackish, locale for hagfish evolution center around the work of Romer and Grove (1935) who concluded from the geological evidence that vertebrates arose in freshwater localities, and that of Homer Smith (1932) who believed that the glomerular kidney, with which hagfish are well provided, evolved as a volume regulatory device to rid the body of excess fluid as a result of water influx in dilute environments. The presence of glomerular kidneys, plus the suggestion that calcium phosphate cannot be deposited in bone in a high ionic medium, are the principle arguments currently upholding vertebrate freshwater origins (Griffith, 1985).

The more recent discovery of agnathan fossils in marine deposits from the Upper Cambrian and Ordovician, together with the fact that fossils associated with freshwater deposits do not occur until the Silurian period, are supportive of a marine origin (Halstead, 1985; Gilbert, 1993). The presence of glomerular kidneys in agnathans is not necessarily a

hindrance to marine origins, but may be viewed as a preadaptation to a freshwater invasion. The kidney of decapod crustaceans, whose marine origin is undisputed, functions as a filtration-reabsorption system, similar to that of the vertebrate. The chief function in the stenohaline marine decapod kidney is the control of the ionic composition of the blood; however, in euryhaline and freshwater crustaceans, the kidney has assumed volume regulatory functions (Morris, 1960; Kirschner, 1979). The hagfish kidney also appears to regulate blood ion concentrations, particularly by controlling divalent ion concentration (Hardisty, 1979). The presense of ionocytes (cells morphologically similar to the ion-transporting chloride cells of higher fish) in the gills of hagfishes (Mallatt and Paulsen, 1986; Elger, 1987) are hypothesised as originating for acid-base regulation in a marine environment and subsequently taking on ionoregulatory functions (Evans, 1984). Although the question of the environment of the first vertebrates can never be resolved satisfactorily, the hypothesis for a marine origin of the Agnatha, and hence the vertebrates, appears to be fairly robust.

Whatever the environmental origin of the vertebrates, it is now clear that hagfish diverged from the main vertebrate line somewhat in excess of 500 MYA (Forey and Janvier, 1993). The assumption can be made that features shared by both the hagfish and higher vertebrates are ancestral; hagfish, although modern and highly derived fishes, provide a unique study group in which to examine primitive vertebrate characters and their subsequent evolution. However, because hagfish have been evolving independently from other vertebrates for at least 500 million years, caution should be exercised in the interpretation of uniquely hagfish characteristics since these may be derived and not primitive. As indicated above, much research has been devoted to the osmotic status of hagfish and their place in the evolution of osmoregulatory capabilities in vertebrates. The present study examines natriuretic peptide receptors (NPRs) in the gill, kidney, and aortae of the Atlantic hagfish, Myxine glutinosa to determine the receptor characteristics shared by both gnathostomes and hagfish, leading to an assessment of the primitive vertebrate

condition. Natriuretic peptides are a family of peptide hormones that regulate blood volume and pressure in mammals and for which there is increasing evidence of a role in fish osmoregulation (Evans, 1990). The gills and kidneys have been selected for examination because they are important osmoregulatory structures in higher fishes (Evans, 1993).

Natriuretic Peptides and their Receptors

In 1956, Kisch discovered the presence of granules in guinea pig atrial myocytes. Subsequently, it became apparent that these granules resembled storage granules found in typical peptide hormone-producing cells (de Bold et al., 1978). However, it was not until de Bold et al. (1981) injected supernatants of atrial tissue into anaethetized rats and observed a potent urinary diuresis and natriuresis, and concomitant decrease in blood pressure, that a function was postulated for the putative atrial peptide hormone. Since then, research into the atrial factor has escalated and the heart as an endocrine organ secreting natriuretic peptides is firmly established (Rosenzweig and Seidman, 1991).

It is now clear that natriuretic peptides (NPs) are a family of peptide hormones that function in salt and water homeostasis in mammals by primary and secondary effects on the heart, vasculature, kidneys, adrenals, and central nervous system (CNS; Genest and Cantin, 1988; Samson and Quirion, 1990; Brenner at al., 1990; Rosenzweig and Seidman, 1991; Ruskoaho, 1992). The members of this family are: atrial, or A-type, natriuretic peptide (ANP), which is synthesized mainly in atrial myocytes but also to a lesser extent in the ventricle, aortic arch, lung, kidney, adrenals, eye, gastrointestinal tract, thymus, and the CNS (Ruskoaho, 1992); brain, or B-type, natriuretic peptide (BNP), which was initially isolated from porcine brains (Sudoh et al., 1988) but has subsequently been identified in cardiac tissue (Nakao et al., 1990. Hosoda et al., 1991); C-type natriuretic peptide (CNP), which is found in the CNS of mammals (Sudoh, 1990) and in the hearts and brains of fishes (Price et al., 1990; Schofield et al., 1991; Suzuki et al.,

1991; Suzuki et al., 1992); and ventricular natriuretic peptide (VNP), which has been isolated from the cardiac ventricle of eels (Takei et al., 1991). ANP has also been isolated from the hearts of eels (Takei et al., 1989), and a BNP-like peptide has been isolated from eel brain (Takei et al., 1990).

The actions of NPs serve to acutely and chronically reduce blood pressure by decreasing cardiac output, reducing peripheral vascular resistance (partly by relaxation of vascular smooth muscle) and by decreasing intravascular volume. In addition, blood volume is reduced by an increase in glomerular filtration rate and by the potent diuretic and natriuretic effects of NPs in the kidney, and secondarily by inhibiting the release of aldosterone from the adrenals and renin from the juxtaglomerular cells (Brenner at al., 1991). In the brain, NPs have been found in the paraventricular nuclei, which synthesize vasopressin, and the anteroventral region of the third ventricle, which is implicated in blood pressure control (Brenner at al., 1991). NPs are also present in the hypothalamus and the anterior pituitary suggesting neuroendocrine or paracrine control in these areas. Some of the functions of NPs in the CNS involve the control of water intake, salt preference, and inhibition of vasopressin secretion (Samson, 1990).

These peptides share a 17-member ring formed from an intrachain disulfide bond between two cysteine residues. Ten of the seventeen amino acids of the ring are conserved among NPs and the intact ring is essential for biological activity. Each NP is a separate gene product (Rosenzweig and Seidman, 1991). The active form of circulating ANP ($_{99-126}$, or alternatively named $_{1-28}$) is a 26 amino acid peptide which is cleaved from proANP ($_{1-126}$), which in turn has been produced from preproANP, a 152 amino acid precursor. PreproANP is translated from ANP mRNA, which has been transcribed from the ANP gene containing three exons and two introns. ANP_{1-28} has a carboxy- and amino-terminal tail protruding from the ring. ANP amino acid sequences are highly conserved in mammals. BNP is the most divergent of the peptide family and the active form varies between 26 and 45 amino acids in length. The differences in length are the result of

extensions at the amino-terminal. The gene structure and peptide processing of BNP are similar to that of ANP. CNP is a 22 amino acid peptide that terminates with the second cysteine residue on the ring so that there is no carboxy-terminal tail present. It is the most highly conserved of the NPs across all species examined so far (Rosenzweig and Seidman, 1991). VNP is a 36 amino acid peptide with an extended carboxy-terminal tail, which makes it unique among the NP family (Takei et al., 1991).

The effects of NPs are mediated through two membrane bound receptor types: particulate guanylate cyclase-linked (GC) receptors (molecular mass approximately 130 kDa) that activate the guanosine 3',5' -cyclic monophosphate (cGMP) intracellular second messenger system (Martin et al., 1989; Koller and Goeddel, 1992); and the 'clearance' receptor (NPR-C, a homodimer of a 65 kDa protein), which is not coupled to guanylate cyclase activity. NPR-C was originally named because it was believed to modulate circulating concentrations of NPs by their removal from the blood (Chinkers and Garbers, 1991; Maack, 1992). Some studies suggest that NPR-C interacts with second messenger systems other than that of cGMP, such as inhibiting adenylate cyclase via a G-protein mechanism, and stimulating phosphoinositol pathways (Levin, 1993). At least two GC receptors have been identified to date: NPR-A and NPR-B with 44 % homology in the extracellular NP binding region and 88 % homology in the cytosolic catalytic guanylyl cyclase domain (Koller and Goeddel, 1992). NPR-A appears to preferentially bind ANP, but will also bind BNP and to a lesser extent CNP; whereas NPR-B binds CNP preferentially (Suga et al., 1992a). GC receptors require an intact cysteine ring for NP binding; however, NPR-C can bind a diversity of NPs and truncated and ring deleted analogues (Brenner et al., 1990). Recent autoradiographical and membrane binding studies have capitalized on the differential binding abilities of the receptors to identify populations of receptor types and subtypes in mammalian CNS, kidney, adrenals, and aortic smooth muscle (eg. Suga et al., 1992a and b; Konrad et al., 1992; Fethiere et al., 1992; Canaan-Kuhl et al., 1992; Brown and Zuo, 1992).

Natriuretic Peptides and Osmoregulation in Fishes

Fish Osmoregulation

Fishes face a variety of salt and water challenges. Freshwater teleosts and freshwater lampreys chronically gain water and lose salt to their environment whereas saltwater species face the opposite problem of salt loading and water loss. Euryhaline species, moving to and from fresh or brackish water and the marine environment, encounter both situations, at first acutely, and then after adaptation, chronically. Elasmobranchs, which with rare exceptions are marine, have plasma that is slightly hypertonic to sea water; plasma salt concentrations, however, are still lower than in sea water, the balance being made up with urea and trimethylamine oxide. Consequently, in the marine environment, elasmobranchs gain salt and also water. Hagfish, in contrast to other fishes, are exclusively marine and have blood that is isotonic to sea water; as a result they face no osmotic challenges (Evans, 1993).

The mechanisms of fish osmoregulation have recently been reviewed by Evans (1993). In fresh water (FW), fish maintain osmotic homeostasis through branchial salt uptake, renal salt reabsorption and urinary water loss. Branchial salt uptake is via Na^+/H^+ and Cl^-/HCO_3^- exchange so that salt uptake is coupled with the excretion of ions functional in acid/base balance. The cell type in which these exchanges are occurring has not been definitively demonstrated. However, a study by Perry and Laurent (1989) showed a proliferation of chloride cells in the gills of trout exposed to artificial FW with a low salt concentration, suggesting that the chloride cells may be responsible. In salt water, water loss is balanced by drinking salt water and the active uptake of salt and consequent passive influx of water in the gut. The excess salt is subsequently excreted across the gill via the chloride cells which are believed to have $Na^+/K^+/2Cl^-$ cotransporters located on the basolateral membrane and Cl channels on the apical membranes. Sodium follows the electronegative gradient thus generated via leaky paracellular junctions. The $Na^+/K^+/2Cl^-$

cotransporter is also the mechanism of salt uptake by the gut, however, the cotransporter is located on the gut lumenal (apical) membrane, rather than basolaterally. Elasmobranch water gain is balanced by urinary loss and excess salt is excreted via the rectal gland, utilizing the $Na^+/K^+/2Cl^-$ cotransporter, with the gills possibly also aiding in salt secretion (Evans, 1993). Salt and water balance in fishes is under the control of various hormones. In FW, it appears that arginine vasotocin and prolactin are important, whereas in sea water (SW) cortisol and the renin-angiotensin system are critical. Little is known about the physiological control of salt and water in hagfish (for review see Wendelaar Bonga, 1993).

Natriuretic Peptides in Fishes

Since the role of ANP in mammalian salt and water balance was discovered, interest developed in the possible action of NPs in fish osmoregulation (Evans, 1990; Evans and Takei, 1992; Evans, 1994 in press). Immunohistochemical and radioimmunoassays indicate that all classes of fishes have NP systems (Reinecke et al., 1985; Reinecke et al., 1987; Evans et al., 1989; Vallarino et al., 1990; Donald and Evans, 1992; Donald et al., 1992), and various NPs have been isolated and sequenced from both teleosts and elasmobranchs (Takei et al., 1989; Price et al., 1990; Takei et al., 1990; Schofield et al., 1991; Suzuki et al., 1991; Takei et al., 1991 Suzuki et al., 1992).

There is some evidence to suggest that NPs may function as a saltwater hormone in fishes since plasma NP immunoreactivity (NP_{ir}) is greater in some fishes adapted to higher salinities. The euryhaline marine longhorn sculpin and winter flounder, Pseudopleuronectes americanus, adapted to low salinity showed significantly lower plasma NP_{ir} than they did in SW (Evans et al., 1989) and the FW chub (Westenfelder et al., 1988) and the salmon and the trout (Smith et al., 1991) showed increased plasma NP_{ir} during acclimation to increased salinity. This may also be the case in agnathans, at least in euryhaline species, since the acclimation of the lamprey, Petromyzon marinus, to SW was accompanied by an increase in plasma NP_{ir} (Freeman and Bernard, 1990). The eel

(Anguilla japonica); however, appears to be an exception since ANP plasma concentrations decline in higher salinities (Takei and Balment, 1993). A number of studies have found that cardiac extracts and heterologous NPs produce dilation in mammalian and fish vascular smooth muscle (Reinecke et al., 1985; Reinecke et al., 1987; Evans et al., 1989; Evans, 1991; Evans et al., 1992).

Several physiological studies have been performed using heterologous NPs. Of particular note for the present study are the effects on the branchial and renal system. Infusions of cardiac extracts and of large doses of rat ANP produced slight diuresis and a substantial natriuresis in FW trout (Duff and Olson, 1986), and SW toadfish, Opsanus tau (Lee and Malvin, 1987). Salt transport by the gill epithelium is enhanced in vitro in the killifish by ANP application (Schiede and Zadunaisky, 1988); however, the opposite appears to be true in the gut epithelium where salt absorption is inhibited in the winter flounder (O'Grady et al., 1985). NPs also appear to enhance sodium and chloride excretion in perfused shark rectal glands and in cultured rectal gland epithelium (Solomon et al., 1985 and 1992; Forrest et al., 1992; Karnaky et al., 1991 and 1992). Branchial perfusion may also be effected by NPs considering the vasodilation that these peptides produce in fish. If such effects result in increased gill perfusion, osmotic problems could be exacerbated, increasing salt and water losses or gains, depending on the environmental concentration (Evans and Takei, 1992; Evans, 1994, in press).

It is possible that NPs also affect fish osmoregulation secondarily through modulation of other endocrine systems, for example the renin-angiotensin system, or prolactin and cortisol. Arnold-Reed and Balment (1991) showed that ANP increases circulating cortisol levels in SW adapted flounder and stimulates cortisol secretion in SW adapted trout, but not FW adapted trout. In salmon smolts plasma renin activity (PRA) and ANP_{ir} levels rose on transference to SW; however, PRA in salmon parr did not change although plasma ANP_{ir} increased (Smith et al., 1991). Rainbow trout acclimated to SW

for 3 weeks also showed elevated levels of PRA and ANP_{ir} in the plasma. Freshwater trout fed a high salt diet showed elevated ANP_{ir} but not PRA (Smith et al., 1991).

There have been a few studies on NP binding sites in teleosts and elasmobranchs. ^{125}I-ANP binding sites have been found in the heart of the conger eel, Conger conger (Cerra et al., 1992), in the kidney, gills, and heart of two species of antarctic fish (Uva et al., 1993), and in chondrocytes of gill cartilage and parenchymal cells of the secondary lamellae of the Japanese eel, Anguilla japonica (Sakaguchi et al., 1993). ^{125}I-CNP binding has been observed in the rectal gland of the dogfish shark, Squalus acanthias and CNP stimulated cGMP production in this tissue (Gunning et al., 1993). ANP has been found to stimulate cGMP production in isolated rainbow trout nephrons (Perrott et al., 1993). In a series of studies, Duff and Olson (1992) and Olson and Duff (1993) have identified NPR-C-like receptors in the arterio-arterial gill vasculature of the rainbow trout. These binding sites are capable of removing 60 % of ^{125}I-ANP from the circulation in a single pass through the gills. Donald et al. (in press) located ^{125}I-ANP and ^{125}I-CNP binding sites on the afferent and efferent branchial arteries and arterioles of the Gulf toadfish, Opsanus beta. In this study, affinity cross linking experiments showed high (140 kDa) and low (75 kDa) binding sites which suggest a population of NPR-C and GC receptors, similar to those found in mammals.

Hagfish appear to have quite an extensive NP system: the heart, brain, and plasma of the Atlantic hagfish, Myxine glutinosa, have NP-like immunoreactivity (Reinecke et al., 1987; Evans et al., 1989; Donald et al., 1992), and NP binding sites have been located in the archinephric ducts and glomeruli of the kidney, the ventral aorta, and the brain (Kloas et al., 1988; Donald and Toop, unpublished). In addition, rat ANP and CNPs, which dilate the ventral aortic vascular smooth muscle of both the Gulf toadfish, and the dogfish shark, also dilate that of the hagfish, Myxine glutinosa (Evans et al., 1989; Price et al., 1990; Evans, 1991; Evans et al., 1993). As with other fishes, dilation of the ventral aorta could alter the dynamics of gill blood perfusion. The present study was undertaken to extend the

knowledge of NPs in Myxine glutinosa by examining the presence and character of NP binding sites in the gill, kidney, and aorta, using autoradiography, radioligand binding, affinity cross-linking and SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and guanylate cyclase assays; and, as indicated above, to place the information from this study into the context of the evolution of osmoregulation and the natriuretic peptide system in vertebrates.

NATRIURETIC PEPTIDE RECEPTORS IN THE GILLS OF THE ATLANTIC HAGFISH, MYXINE GLUTINOSA

Introduction

Hagfish Gill Morphology

The gross morphology of hagfish gills is considerably different from that of other fishes, to the extent that the homology between the hagfish gill and other fish gills has been questioned (Mallatt, 1984). The hagfish branchial epithelium is derived from endoderm, whereas that of other fishes derives from ectoderm, with the exception of the agnathan lamprey gill, the embryological derivation of which is still unclear (Mallatt, 1984). However, the hagfish shares the same gill tissues and cell types with the higher fishes (Mallatt and Paulsen, 1986). For example, hagfish gill lamellae are lined with pavement cells, basal cells, and ionocytes (structurally similar to the chloride cells of other fishes). There are also typical pillar cells, marginal channels, and cavernous bodies (Mallatt and Paulsen, 1986; Elger, 1987). Mallatt (1984) concludes that the hagfish gill is derived from a more primitive version of the vertebrate gill than the ancestral gnathostome and lamprey gill.

The morphology of hagfish gills is diagrammed in Fig. 2-1. The gill lamellae are contained in ovoid muscular pouches through which water flows countercurrent to the blood; in Myxine glutinosa six gill pouches lie on either side of the esophagus (Fig. 2-1a). There are three main regions inside the gill pouch, afferent, lamellar, and efferent (Fig. 2-1b). The afferent section (relative to blood flow) includes a multilayered epithelium containing ionocytes separated by connective tissue from the arterio-arterial vasculature. The vasculature consists of a network of radial vessels and afferent cavernous tissue

surrounded by smooth muscle. Arranged between the afferent and efferent sections of the gill are the respiratory lamellae. These are characterized by a bilayered epithelium in which ionocytes are present. There is no smooth muscle in this section of the gill. The lamellar portion of the gill is drained by efferent lamellar arterioles and the efferent cavernous tissue. The efferent portion of the gill is also characterized by a multilayer epithelium; however, ionocytes are absent here (Elger, 1987).

Natriuretic Peptides and Fish Gills

The gills are important sites of NaCl transport (either uptake or excretion) in osmoregulating fishes (see General Introduction above, and Evans, 1993). As such, the potential role of NPs in gill hemodynamics and/or salt transport has been investigated. There is some physiological evidence to suggest that NPs dilate gill microvasculature since afferent pressure decreases slightly by the dilation of the arteriovenous pathway when ANP is added to perfused steelhead trout, Salmo gairdneri, gills, and ANP also relaxes epinephrine-stimulated increases in gill vascular resistence (Olson and Meisheri, 1989). These data are corroborated by Evans et al. (1989) who found that branchial resistence decreased when ANP was added to the perfusate in Gulf toadfish, Opsanus beta, perfused head preparations, although this may partly have been the result of dilation of the ventral aorta by ANP. NPs have been shown to dilate the vascular smooth muscle of the ventral aorta in all classes of fish, including hagfish (Reinecke et al., 1985; Reinecke et al., 1987; Evans et al., 1989; Evans, 1991; Evans et al., 1992). Because the gill vasculature is in series with the systemic circulation, all blood leaving the heart passes through the gills via the ventral aorta before travelling to the rest of the body. Presumably, therefore, the vasodilatory effects of NPs on the ventral aorta will modulate perfusion of the gill, increasing the surface area of the blood/water respiratory interface and thus potentially exacerbating net osmotic and ionic gains or losses. It is therefore difficult to interpret the function of NPs on the gill vasulature (Evans et al., 1989; Evans and Takei, 1992).

There have not been many studies on the role of NPs in gill salt transport and the majority of existing information is corollary rather than direct. Schiede and Zadunaisky (1988) demonstrated an increase in chloride secretion in Fundulus heteroclitus opercular preparations, which are rich in chloride cells (the site of NaCl transport). These results suggest that NPs affect the $Na^+/K^+/2Cl^-$ cotransporter, especially because NPs have similar affects on the same transport system in shark rectal gland (Solomon et al., 1985, 1992; Forrest et al., 1992; Karnaky et al., 1991, 1992). ANP inhibited Na and Cl uptake across the intestine of the winter flounder, Pseudopleuronectes americanus (O'Grady et al., 1985) and the eel, Anguilla japonica (Ando et al., 1992). These results are paradoxical since NaCl uptake is reported to occur across the gut epithelium using the same $Na^+/K^+/2Cl^-$ cotransport system as is used in salt extrusion by the gill (Evans, 1993). Whole animal Na efflux was increased by the injection of ANP in chronically cannulated SW adapted flounder, although the mechanism of action is unknown (Arnold-Reed et al., 1991).

The role of NPs in gill function has also been suggested through an examination of NP receptors in this tissue. As has already been stated above, there are two main classes of NPR: the GC-linked receptors, NPR-A and NPR-B; and the 'clearance' receptor, NPR-C. Populations of NPRs have been identified on gill microvasculature. Duff and Olson (1992) and Olson and Duff (1993) have identified receptors in the arterio-arterial gill vasculature of the rainbow trout. These binding sites are capable of removing 60 % of ^{125}I-ANP from the circulation in a single pass through the gills, suggesting, in part, a clearance function for these receptors. Donald et al. (in press) located ^{125}I-ANP and ^{125}I-CNP binding sites on the afferent and efferent branchial arteries and arterioles of the Gulf toadfish, but binding did not appear to be associated with the respiratory epithelium where the chloride cells are concentrated. Affinity cross-linking experiments showed high (140 kDa) and low (75 kDa) molecular mass (M_r)binding sites that suggest a population of NPR-C and GC receptors, similar to those found in mammals. Guanylate cyclase activity was associated with at least some of these NP receptors because cGMP production was augmented in a dose dependent

manner with the addition of increasing concentrations of ANP and CNP. In contrast, NP binding has been associated with the chloride cells of two species of antarctic fish, Chiondraco hamatus and Pagothenia bernacchi (Uva et al., 1993), and in the chondrocytes of gill cartilage of the Japanese eel, Anguilla japonica, and to a lesser extent on the parenchymal cells of the secondary lamellae, a region in which chloride cells are located (Sakaguchi et al., 1993). The evidence from the teleost binding studies do not exclude any of the proposed functions for NPs in gill tissue, so that their role may include both the control of the branchial vasculature and a function in ionic regulation. The localization of NP binding sites in gill cartilage chondrocytes is somewhat puzzling, however. The majority of these receptors appear to be an NPR-C like receptor, but the function of such receptors in chondrocytes and the NPR subtype of the lamellar parenchymal cells are unknown (Sakaguchi et al., 1993). The presence of NPR-C like receptors in the trout and the Gulf toadfish (Duff and Olson, 1992; Donald et al., in press) indicate a clearance function for this receptor subtype, or alternative second messenger system mechanisms, in gill tissue.

The present chapter describes the localization, kinetic parameters, and character of NP binding sites in the gills of the Atlantic hagfish. The study was performed using autoradiography of gill sections and NP radioligand binding assays of isolated gill membrane preparations in order to localize and characterize hagfish gill NPRs; guanylate cyclase assays, to determine the presence of GC receptors; and affinity cross-linking of radiolabelled NPs to receptors followed by SDS-PAGE, to assess the apparent molecular mass of the receptors.

Materials and Methods

Animal Maintenance

Hagfish were collected from the Bay of Fundy and supplied by Huntsman Marine Laboratory, St Andrews, N.B. and maintained in running SW (12 - 14 °C) at the Mount Desert Island Marine Laboratory, Maine; or in 10 °C tanks aerated through charcoal/fiber filters at the University of Florida, Gainesville. Animals were allowed to equilibrate for at least three weeks before experimentation. Hagfish were anaesthetized in MS 222 (1:1000, Sigma, St.Louis, MO) before dissection. Animals were killed by severence of the spinal chord caudad to the brain which was then removed.

Autoradiography

After dissection, tissues were freeze mounted in Tissue Tek (Miles Inc. Elkhart, Indiana) in a microtome cryostat (Minotome, IEC, Massachusetts). Eighteen micrometer sections were cut and mounted on gelatin-chromium aluminium coated slides before being dried overnight under vacuum at 4°C. The sections were stored in sealed boxes at -20 °C until used.

Sections were preincubated for 15 min at room temperature (22-24 °C) in 50 mM Tris-HCl buffer (pH 7.4), 50mM NaCl, 5mM $MgCl_2$, 0.1 % bovine serum albumin (BSA), and 0.05% bacitracin. The sections were then incubated for 90 min in the same buffer supplemented with 4μg/ml of leupeptin, 2μg/ml chymostatin, 2μg/ml pepstatin, 10^{-6} M PMFS (phenylmethylsulfonylfluoride), and rat (3-[^{125}I]iodotyrosol^{28}) atrial natriuretic peptide (2000 Ci/mmol; Amersham, Illinois), or human, porcine, rat (^{125}I-[Tyr^{0}]) C-type natriuretic peptide-22 (1500Ci/mmol; Peninsula Laboratories, California). Nonspecific binding was determined in adjacent sections in the presence of 10^{-6} M unlabelled rat 3-28 ANP (rANP, Bachem, California) for ^{125}I-ANP incubated sections, and 10^{-6} M porcine

CNP (pCNP; Bachem, California) for ^{125}I-CNP incubated sections. Displacement of specific binding was also determined in the presence of pCNP (^{125}I-ANP labelled sections), rANP (^{125}I-CNP labelled sections), and rat des[Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}]ANP-(4-23)-NH_2 (C-ANF; Bachem, California), a truncated ANP that binds only to NPR-C in mammals (Maack et al., 1987). Following incubation, the slides were washed (2 x 10 min at 4 °C) in 50 mM Tris-HCl buffer, fixed for 20 min in 4% formaldehyde in 0.1M phosphate buffer (pH 7.4, 4°C), washed in 0.1M phosphate buffer (pH 7.4, 4°C), and then in distilled water (1 min), dehydrated through alcohols and dried overnight at 60 °C. Sections were apposed to Hyperfilm-ßmax (Amersham, Illinois) for 5 days at room temperature. The film was processed using Kodak GBX developer (4 min), rinsed in water (2 min), and fixed with Kodak GBX fixer (5 min).

For examination of binding sites with light microscopy, some sections were dipped in nuclear track emulsion (Kodak NTB.2) at 43 °C. After drying the sections were stored for ten days at 4 °C, and then developed in Kodak D 19 (3 min), washed in water, and fixed in Kodak Rapid Fixer diluted 1:1 (7 min). Subsequently they were stained in 1% toluidine blue, examined with an Olympus BH-2 microscope, and photomicrographs made with a Wild Leitz MPS 46 Photoautomat camera on Kodak T-max 100 black and white film.

Membrane Preparation

Gill membranes were prepared from individual hagfish for saturation and competition binding studies, affinity cross-linking followed by SDS-PAGE, and for guanylate cyclase assays. The gill pouches were removed from anaesthetized hagfish and placed in a 50 ml centrifuge tube in 5 ml of ice-cold 50 mM Tris-HCl and 1mM $NaHCO_3$ (pH 7.4) and quickly homogenised with a Tissue-Tearor (Biospec, Bartlesville, Oklahoma). The homogenate was diluted with 5 ml 50 mM Tris-HCl, 1mM EDTA and 1mM $MgCl_2$ (pH 7.4) and centrifuged at 800 x g for 10 min at 4 °C. The supernatant was

collected and centrifuged at 30,000 x g for 20 min. The pellet was washed with 50mM Tris-HCl (pH 7.4) and 250 mM sucrose and resuspended in 400 µl of the same sucrose buffer. Protein concentration was determined with a BCA protein assay kit (Pierce) calibrated against BSA standards. Membranes were stored at -70 °C until used.

Radioligand Binding Assays

For the ANP saturation curves, 75 µg of gill membrane protein was incubated in 250 µl of incubation buffer (the same as used for sections, see above) in the presence of increasing concentrations (5 - 300 pM) of [^{125}I]-rANP. Binding reactions were performed for 90 min at 23 °C. The reaction was terminated by the addition of 2 ml of ice-cold 50 mM Tris-HCl (pH 7.4) and filtered through 1% polyethylenimine-treated Whatman GF/C filters. Filters were washed with 5 ml of 50 mM Tris-HCl (pH 7.4), and the radioactivity was measured in a Beckman Gamma counter with 78% efficiency. For competition binding studies, 75 µg of gill membrane protein was incubated in 250 µl of the incubation buffer and 25 pM of [^{125}I]-rANP, or [^{125}I]-pCNP with the unlabelled peptides rANP, C-ANF, and pCNP present in increasing concentrations (10^{-12} - 10^{-6} M). Binding reactions were performed as above.

Affinity Cross-Linking

Hagfish gill membranes were isolated as described above and 100-125 µg of protein incubated in incubation buffer with 0.25 nM iodinated peptide in the presence or absence of excess unlabelled rANP, pCNP, or C-ANF. The final incubation volume was 250 µl. Affinity cross-linking was performed according to Martin et al. (1989). Following incubation, the covalent cross-linking agent disuccimidyl suberate (DSS; Pierce) in dimethylsulfoxide was added to a final concentration of 1 mM and the reaction was mixed gently for 20 min at 23 °C. The cross-linking reaction was stopped by the addition of an equal volume of quench buffer (400 mM EDTA and 1M Tris-HCL, pH 6.8). Membranes

were centrifuged in an eppendorf centrifuge at 13,000 x g for 20 min to separate unbound hormone, and the pellet resuspended in 30 µl of sample buffer for SDS-PAGE, containing 62.5 mM Tris base, 2 % SDS, 5 % glycerol, 0.01 % bromophenol blue, 2 % ß-mercaptoethanol, pH 6.8, and then boiled for 4 min. Samples, including one of molecular mass markers (30,000 - 200,000 kDa), were loaded onto a 7.5 % unidimensional polyacrylamide slab gel and electrophoresed at 200V. Gels were stained with Coomassie brilliant blue (Bio-Rad), dried, and apposed to Hyperfilm MP (Amersham) with intensifying screens for 1-2 weeks at -70 °C. Films were developed as for section autoradiography. Molecular weights of subsequent bands were determined as predicted values from the regression equations of the negative log of relative mobility versus molecular mass standards for each gel.

Guanylate Cyclase Assays

Gill membranes were isolated as above and used immediately. For determination of guanylate cyclase activity, 50 µg of gill protein was added to 50 mM Tris.HCl, 2 mM isobutyl methylxanthine (IBMX), 10 mM creatine phosphate, 1000 U/ml creatine phosphokinase, 4 mM $MnCl_2$, 1 mM GTP and increasing concentrations of rANP, pCNP, and C-ANF in a final volume of 100 µl. A second experiment was performed to establish the effects of C-ANF on guanylate cyclase stimulation by rANP; for this protocol, increasing concentrations of rANP were added to the reaction mixture in the presence of 1 µM C-ANF. The basal rate of cGMP generation was determined in tubes without ligand. The incubations were performed for 15 min at 24 °C, and were terminated by the addition of 4 mM EDTA. The tubes were boiled for 3 min and centrifuged at 2,300 x g for 15 min. The supernatant was collected and frozen and the cGMP content was determined by radioimmunoassay (cGMP RIA kit, Amersham, Arlington Heights, Illinois).

Data Analysis

The values of equilibrium dissociation constant, Kd, and the total number of binding sites, B_{max} , were determined from the saturation binding data using EBDA and LIGAND computer programs (McPherson, 1985). Additional statistics were computed using the Statview SE program (Abacus Concepts, 1988). Data are presented as means ± 1 standard error.

Results

Autoradiography

Both ^{125}I-ANP and ^{125}I-CNP specific binding were observed on the respiratory lamellae of the hagfish gill (Figs. 2a and 3a); specific binding of radioligands was displaced by both 1 μM rANP and 1μM pCNP (Figs. 2b,c and 3b,c). One micromolar C-ANF did not completely displace ^{125}I-ANP binding but displaced virtually all ^{125}I-CNP binding (Figs. 2d and 3d). Examination of radiotrack emulsion-dipped sections indicated that specific binding sites for both radioligands were scattered generally in the lamellar region over the thin bilayered epithelium (Figs. 4a and 4b, ^{125}I-CNP binding only shown, ^{125}I-ANP binding was similar). The exact cell types to which the radioligands were binding could not be determined. Specific binding sites were not observed in either the afferent or efferent gill regions.

Radioligand Binding Assays

Saturation binding

Both ^{125}I-ANP and ^{125}I-CNP bound specifically and saturably to hagfish gill membranes. Maximum binding for both radioligands was reached by 200 pM (Figs. 5a and 6a). EBDA and LIGAND analysis indicated that ^{125}I-ANP binding fit a single site

model with an apparent Kd of 15.4 ± 1.6 pM and a B_{max} of 45.9 ± 3.0 fmol/mg protein, or alternatively, multiple sites with equal affinities (Fig. 5b). Analysis of the ^{125}I-CNP binding site indicated a two site model with a high and low affinity site (Fig. 6b). The high affinity site was not statistically different from the ^{125}I-ANP site, with an apparent Kd of 12.9 ± 4.7 pM and a B_{max} of 23.4 ± 6.5 fmol/mg protein. The low affinity site had an apparent Kd and B_{max} of 380 ± 80 pM and 120 ± 21 fmol/mg protein.

Competition Binding

One nanomolar unlabelled rANP, and 20 and 30 nM unlabelled pCNP and C-ANF, respectively, competed for 50 % of ^{125}I-ANP specific sites. One hundred nanmolar rANP bound virtually all ANP sites. One micromolar pCNP and 1μM C-ANF competed for all but 5 % and 10 % of binding sites respectively (Fig. 7). One tenth nanomolar rANP and pCNP, and 8 nM C-ANF competitively inhibited 50 % of ^{125}I-CNP specific binding. One nanomolar rANP, 10 nM pCNP, and 1μM C-ANF bound 100 % of ^{125}I- CNP specific sites (Fig. 8). Rat ANP and pCNP competed equally for ^{125}I-CNP binding sites above 50 % binding. However, below 50 % binding rANP was a more effective competitive inhibitor than pCNP, suggesting that these sites are low affinity CNP binding sites (in accordance with the saturation analysis above) that bind ANP with greater affinity.

Affinity Cross-Linking and SDS-PAGE

Affinity cross-linking followed by SDS-PAGE under reducing conditions of ^{125}I-ANP and ^{125}I-CNP binding to gill membranes indicated an apparent single binding site with a mean M_r of 150 ± 3 kDa for ^{125}I-ANP and 153 ± 16 kDa for ^{125}I-CNP (Fig. 2-9 a, b; lane 1). Binding was prevented by the addition of 0.1 μM rANP and pCNP to the incubation reaction (Fig. 2-9 a, b; lanes 2 and 3). The addition of 0.1μM C-ANF did not completely inhibit ^{125}I-ANP binding (Fig. 9 a; lane 4), although in other experiments (not shown here) visible binding was prevented by 1 μM C-ANF. ^{125}I-CNP binding was completely blocked by the addition of 0.1 μM C-ANF (Fig. 9 b; lane 4). For neither

iodinated peptide did a second lower band appear, contrasting with mammals and teleosts that show a lower band indicative of the NPR-C homodimer breaking into the monomeric species under reducing conditions (Martin et al., 1989; Donald et al., in press).

Guanylate Cyclase Assays.

The basal cGMP accumulation rate was 2.9 ± 0.4 pM cGMP/mg protein/min. Both rANP and pCNP stimulated cGMP production in a dose dependent manner; 0.1 nM rANP and above, and 10 μM pCNP and above, significantly stimulated cGMP production above basal rate (Fig. 10a). A maximum rANP stimulated rate of 6.2 ± 1.6 pM cGMP/mg protein/min was reached by 0.1 μM rANP. The maximum pCNP stimulated rate, 4.2 ± 0.6 pM cGMP/mg protein/min, was approached at 1 μM pCNP. C-ANF did not stimulate cGMP production at any concentration.

The effect of 1.0 μM C-ANF on rANP stimulated rates of cGMP production was twofold (Fig. 10b). Firstly, cGMP production rates at 0.1 nM and 1.0 nM rANP were significantly higher in treatments with 1 μM C-ANF than in those with rANP alone. Secondly, cGMP production was significantly lower at 1.0 μM rANP in treatments with additional C-ANF than in those with rANP alone.

Discussion

The present study shows that there are both ANP and CNP receptors on the lamellar epithelium of the gills of M. glutinosa (Figs. 2,3, and 4). Because the epithelium in the lamellar region does not contain smooth muscle, receptors may be involved in epithelial cell function (perhaps ionocyte function), or in NP clearance from the circulation, rather than in the regulation of blood flow. Although it is unlikely that NPs are directly regulating blood flow in this region, the presence of NPRs in the ventral aorta (Kloas et al., 1988; Chapter 3, this study) and the ability of NPs to dilate this tissue (Evans 1991; Evans et al., 1993), suggest that NPs can regulate blood flow to the branchial vasculature as a

whole. The presence of NP receptors in the gill agrees with teleost studies; binding is predominantly located on the chloride cells of two species of antarctic fishes, Chionodraco hamatus and Pagothenia bernacchii (Uva et al., 1993), and Broadhead et al. (1992) suggest that binding occurs on chloride cells of the eel, Anguilla anguilla. In the Japanese eel, Anguilla japonica, NP binding is localized mainly on chondrocytes of gill cartilage and on parenchymal cells, which include chloride cells, of the secondary lamellae (Sakaguchi et al., 1993). Other studies have shown binding in the arterio-arterial gill vasculature in trout where receptors appear to perform a clearance function (Olson and Duff, 1993) and on the afferent and efferent branchial arteries and arterioles of the Gulf toadfish, (Donald et al., in press). In the latter study, affinity cross linking experiments showed high (140 kDa) and low (75 kDa) M_r binding sites which suggest a population of NPR-C and guanylate cyclase receptors, similar to those found in mammals.

Saturation analysis indicates that there are saturable ANP and CNP binding sites in the gill (Figs. 5 and 6). Interpretation of the Scatchard plots suggest that ANP binds to at least one high affinity site, or possibly more than one site with equal affinities; whereas CNP binds to two sites of differing affinities. The competition data demonstrate that ANP and CNP are capable of competing for the binding sites of both radioligands, but with different efficiencies. CNP is less able to compete with ANP for ^{125}I-ANP sites (Fig. 6), but ANP and CNP compete equally well for ^{125}I-CNP sites (Fig. 7), suggesting that there are two ANP sites, one which binds ANP in preference to CNP (the low affinity CNP site, hereafter referred to as Site 1), and one which binds ANP and CNP with equal affinity (the high affinity CNP site, hereafter referred to as Site 2). In addition, the similarity in the upper 50 % of the competition curves of rANP and pCNP for ^{125}I-CNP binding sites (Fig. 8) is consistent with the presence of a high ANP/CNP affinity site, Site 2, whereas the inability of pCNP to compete for ^{125}I-CNP as effectively as rANP in the lower 50 % of the curves suggests the presence of a high affinity ANP/low affinity CNP site, Site 1. A similar situation was found in shark rectal gland competition studies where CNP, instead of

ANP, displaced specific binding more readily than ANP below 50 % binding on the curve (Gunning et al., 1993). The stimulation of cGMP production by ANP (Fig. 10a), and to a lesser extent by CNP, indicates that Site 1 is probably coupled to GC activity. Site 1 appears to correspond to the NPR-A of mammals (Fig. 11, Site 1), since mammalian GC-linked NPR-A receptors bind ANP with much greater affinity than CNP; however, in cells cultured specifically to express the mammalian NPR-A, CNP failed to stimulate cGMP production, unlike Site 1 in the present study (Koller and Goeddel, 1992). There is no evidence in the hagfish gill of a GC-linked NPR-B like receptor that preferentially binds CNP (Koller and Goeddel, 1992).

C-ANF competitively inhibits the majority of both ^{125}I-ANP and ^{125}I-CNP binding (Figs. 6 and 7), but it is unclear to which site this NP analogue preferentially binds. Because it appears to only partially displace ^{125}I-ANP but all of ^{125}I-CNP from tissue sections, it is tempting to suggest that C-ANF binds mainly to the Site 2 receptor (Figs. 2d and 3d). C-ANF has been constructed to bind specifically to the clearance (NPR-C) receptor in mammals (Maack et al., 1987); however, whether this specificity for NPR-C is maintained in other vertebrate classes is unknown. There is some evidence from this study to suggest that C-ANF, while it binds preferentially to the Site 2 receptor, also binds, to some extent, to the Site 1, NPR-A like receptor. Guanylate cyclase activity is stimulated above control levels at lower concentrations of rANP with the addition of 1 μM C-ANF (Fig. 10b), indicating that the action of C-ANF blocking Site 2 receptors increases the effective concentration of rANP. However, at 1.0 μM rANP the cGMP accumulation rate appears to be inhibited below control levels when 1.0 μM C-ANF is also present suggesting the additional binding of C-ANF to some of the Site 1 GC receptors. It is clear that, even with C-ANF binding to some of the GC receptors, C-ANF still does not stimulate GC activity, hence the observed decrease in cGMP production at 1.0 μM rANP.

Both receptor types in the hagfish gill have an apparent M_r of approximately 150 kDa (Fig. 9). This molecular mass is slightly heavier than the M_r of the mammalian NPR-

A, and the homodimer form of the NPR-C, which both appear at approximately 130 kDa. In the presence of reducing conditions mammalian NPR-C, and toadfish NPR-C-like receptors, separate into the monomeric species and are visible as lower molecular mass bands at 65 kDa (mammals, Brenner et al., 1990) and 75 kDa (toadfish, Donald et al., in press). Sakaguchi et al. (1993) discovered a 68 kDa band under reducing conditions in Japanese eel gill membranes; biochemical characterization demonstrated that this receptor was of the NPR-C type. The absence of a lower band in hagfish gills strongly suggests that, unlike in mammals and teleosts, the Site 2 receptor is not a homodimeric NPR-C type. Consequently, it appears unlikely that the Site 2 receptor is homologous in structure to the NPR-C of mammals (Fig. 11, Site 2). It is also unlikely that this receptor is linked to GC activity because CNP did not stimulate cGMP production as effectively as ANP, and C-ANF failed to stimulate it at all (Fig. 10a). Whether the Site 2 receptor functions as a clearance receptor has yet to be determined; however, it is possible that the hagfish gill contains receptors to modulate circulating NP concentrations, since teleost gills perform a clearance function for NPs and other humoral factors (Olson and Duff, 1993). It is also unknown whether the Site 2 receptor is linked to other second messenger pathways, as is now becoming evident for the mammalian NPR-C (Arnand-Srivastava and Trachte, 1993; Levin, 1993).

The present study extends NP function to the gills of hagfish. The absence of smooth muscle in the lamellar epithelium, as mentioned above, suggests that control of blood flow is not a function of the Site 1 receptor in this tissue. However, some mammalian studies have shown potent cGMP stimulation in tissues where binding sites were not detected (Leitman and Murad, 1990). It is possible, therefore, that NP binding could occur on smooth muscle cells, and thus control local blood flow, in other regions of the hagfish gill without being detected by autoradiography. Nevertheless, NP binding in the lamellar region of the gill must contribute to other functions. It is probable that the Site 2 receptors are concerned, at least in part, with clearance of NPs from the circulation.

The location of NP receptors on the lamellar epithelium where ionocytes are found invites speculation on the possible effects of NPs on this cell type. Ionocytes are mitochondrion-rich cells similar morphologically to the chloride cells of higher fish (Elger, 1987). Chloride cells function in salt excretion in marine teleosts (Evans, 1993), an inappropriate function for the osmoconforming hagfish. However, a recent study suggests that salt uptake by freshwater trout may be facilitated by chloride cells (Perry and Laurent, 1989). Branchial salt uptake in freshwater teleosts is believed to occur via Na^+/H^+ and Cl^-/HCO_3^- exchange, mechanisms that are also believed to be present in the hagfish gill since H^+ and base extrusion from whole hagfish depends on the presence of Na^+ in the environmental water for H^+ efflux, and the presence of Cl^- for base efflux (Evans, 1984). These mechanisms probably developed for acid/base regulation, which, unlike salt regulation, would be valuable for the hagfish in encountering anaerobic environments during the course of its burrowing life style. There is some evidence to suggest that the ionocytes may be responsible for these acid/base exchanges in hagfish. Both Na/K-ATPase and carbonic anhydrase activity have been localized in the ionocytes of the Pacific hagfish, Eptatreus stouti, (Mallatt et al, 1987; Conley and Mallatt, 1988). Carbonic anhydrase catalyzes the reaction of CO_2 and H_2O to carbonic acid that dissociates into H^+ and HCO_3^-, and has been observed in the branchial cells of water breathing animals (Cameron, 1989). High levels of Na/K/ ATPase have also been implicated in the function of Na^+ transport; its presence on branchial basolateral membranes is believed to move intracellular Na^+ into the extracellular fluid following apical Na^+ uptake (Kirschner, 1979). It has been suggested that the presence of Na^+/H^+ and Cl^-/HCO_3^- exchange mechanisms in the osmoconforming agnathan ancestors of FW fishes were a 'preadaption' for osmoregulation in FW (Evans, 1984, 1993); it would therefore be interesting if NPs, an important hormone in salt and water balance in higher vertebrates, were found to be functional in the control of acid-base regulation in the hagfish gill.

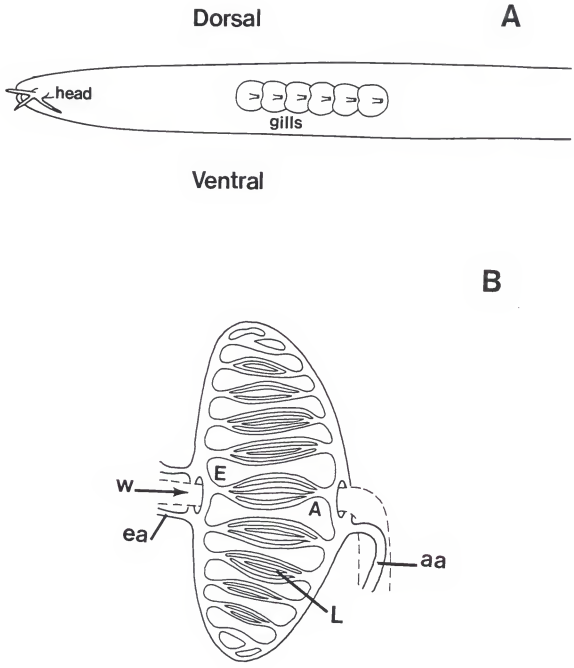

Figure 2-1. Diagram of the position of gills in the hagfish body, and longitudinal section through a single gill, indicating major gill regions.
A. Position of gills in body of hagfish, lateral view.
B. Longitudinal section through a single gill. W, arrow, indicates direction of water flow through gill; aa, afferent arteriole; ea, efferent arteriole; A, afferent region of gill relative to blood flow; E; efferent region of gill relative to blood flow; L, lamellar region of gill.

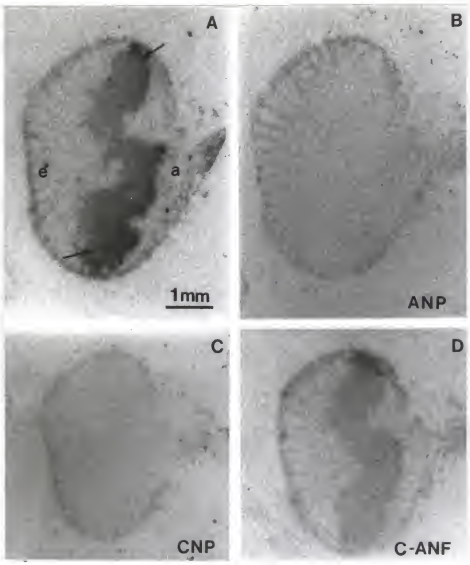

Figure 2-2. Autoradiographs of four serial longitudinal sections of a single hagfish gill pouch incubated with ^{125}I-ANP alone (A) or in the presence of various unlabelled NPs (C-D).
A. Specific binding in the respiratory lamellar region of the gill (arrows); a, region of the afferent vasculature supplying the lamellae; e, region of the efferent vasculature draining the lamellae.
B. Displacement of the specific binding on the lamellae by 1μM rANP revealing the level of non-specific binding.
C. Displacement of specific binding by 1μM pCNP.
D. Partial displacement of the specific binding by 1μM C-ANF.

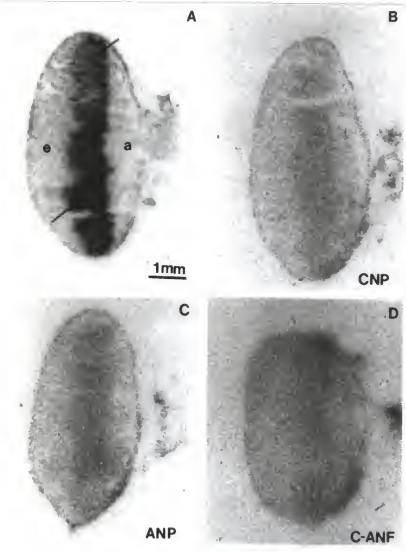

Figure 2-3. Autoradiographs of four serial longitudinal sections of a single hagfish gill pouch incubated with ^{125}I-CNP alone (A) or in the presence of various unlabelled NPs (C-D).
A. Specific binding in the respiratory lamellar region of the gill (arrows); a, region of the afferent vasculature supplying the lamellae; e, region of the efferent vasculature draining the lamellae.
B. Displacement of the specific binding on the lamellae by 1μM pCNP revealing the level of non-specific binding.
C. Displacement of specific binding by 1μM rANP.
D. Displacement of the specific binding by 1μM C-ANF.

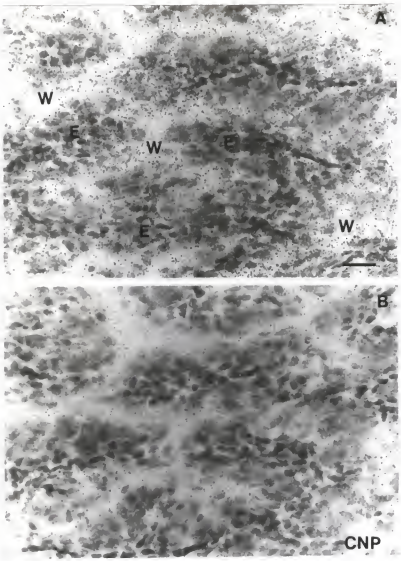

Figure 2-4. Light micrograph of longitudinal gill sections dipped in X-ray sensitive emulsion showing the distribution of specific binding in the lamellar region.
Scale bar: 11.8 mm = 25 μM.
A. ^{125}I-CNP specific binding to the lamellar epithelial cells (E), specific binding indicated by the density of the silver grains; W indicates water channels. Orientation and magnification are the same in B.
B. ^{125}I-CNP with the addition of 1μM pCNP showing the level of non-specific binding; the density of silver grains is reduced. Results (not shown) were similar for ^{125}I-ANP binding.

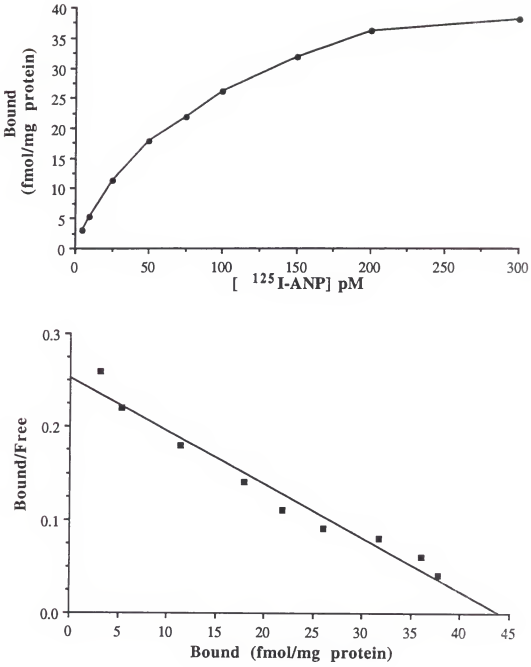

Figure 2-5. Saturation analysis of ^{125}I-ANP specific binding to gill membranes.
A. Example of a typical saturation plot of ^{125}I-ANP specific binding.
B. Scatchard plot of same data showing linear distribution.

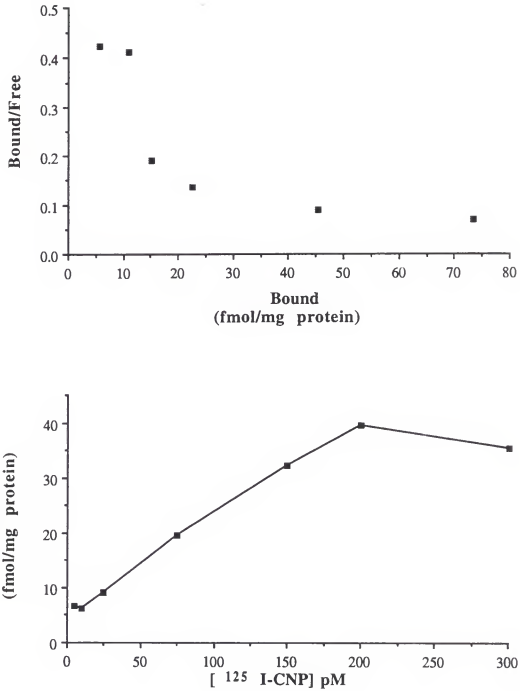

Figure 2-6. Saturation analysis of ^{125}I-CNP specific binding to gill membranes.
A. Example of a typical saturation plot of ^{125}I-CNP specific binding.
B. Scatchard plot of same data showing non-linear distribution.

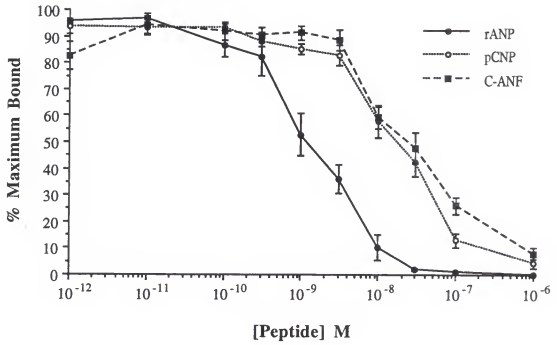

Figure 2-7. Competition study indicating the relative abilities of rANP, pCNP, and C-ANF at increasing concentrations to compete for ^{125}I-ANP specific binding sites. 25 pM ^{125}I-ANP were added to each membrane incubation reaction. Each point is the mean ± SE of gill membrane preparations from 10 separate hagfish.

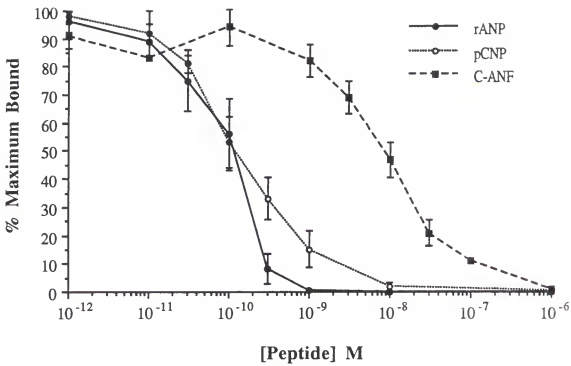

Figure 2-8. Competition study showing the relative abilities of rANP, pCNP, and C-ANF at increasing concentrations to compete for ^{125}I-CNP specific binding sites. 25 pM ^{125}I-CNP were added to each membrane incubation reaction. Each point is the mean ± SE of gill membrane preparations from 5 separate hagfish.

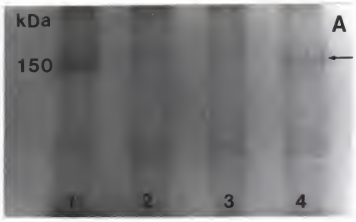

Figure 2-9. Autoradiograph of SDS-PAGE of hagfish gill NP binding sites affinity cross-linked with iodinated NPs under reducing conditions.

A. Cross-linked with ^{125}I-ANP. Specifically labelled band (lane 1) indicates an apparent M_r of 150 kDa. Cross-linking of radiolabelled ligand was inhibited by the presence of 0.1 μM rANP (lane 2), 0.1 μM pCNP (lane 3), and only partially inhibited in the presence of 0.1 μM C-ANF (small arrow, lane 4).

B. Cross-linked with ^{125}I-CNP. Specifically labelled band (lane 1) indicates an apparent M_r of 153 kDa. Cross-linking of radiolabelled ligand was inhibited by the presence of 0.1 μM rANP (lane 2), 0.1 μM pCNP (lane 3), and 0.1 μM C-ANF (lane 4).

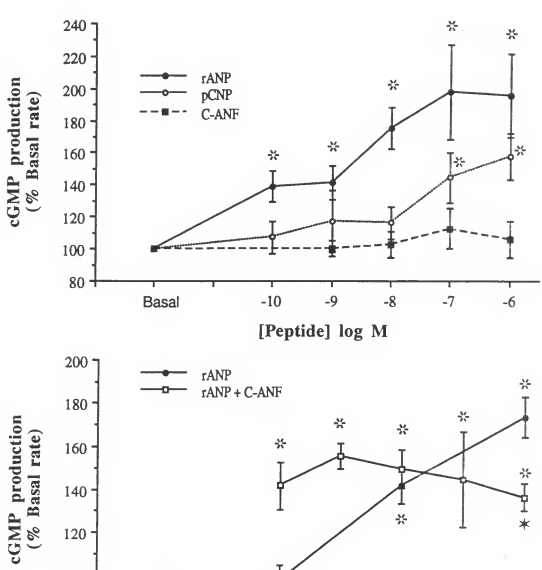

Figure 2-10. Effects of natriuretic peptides on cGMP production rate in hagfish gill membrane preparations.
A. Relative effects of increasing concentrations of rANP, pCNP, C-ANF on cGMP production rate. Data points are mean ± SE of membrane preparations from 5 individual hagfish. ✲Significant increase above basal levels ($p < 0.05$).
B. Effects of increasing concentrations of rANP on cGMP production rate in the presence and absence of 1μM C-ANF. Data points are means ± SE of membrane preparations from 4 individual hagfish. ✲Significant increase above basal levels. ✶Significantly lower than cGMP production rate for rANP alone ($p < 0.05$).

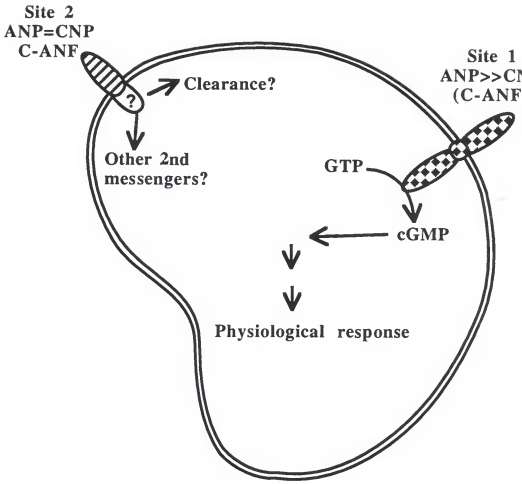

Figure 2-11. Model of NP receptors in hagfish gills.
Site 1: NPR-A like receptor, binds ANP in preference to CNP; GC-linked.
Site 2: ANP/CNP receptor, binds ANP and CNP with equal affinity, may be functionally homologous with mammalian NPR-C; not coupled with GC activity.

NATRIURETIC PEPTIDE RECEPTORS IN THE DORSAL AND VENTRAL AORTAE, AND THE KIDNEYS OF THE ATLANTIC HAGFISH, MYXINE GLUTINOSA

Introduction

Natriuretic Peptides and the Mammalian Kidney

The discovery by deBold et al. (1981) of the potent diuretic and natriuretic effects of atrial extracts on the kidney led to the swift isolation and sequencing of ANP (Atlas et al., 1984; Currie et al., 1984) and, subsequently, to the identification of the other members of the NP family (Sudoh et al., 1988; Sudoh et al., 1990). ANP enhances glomerular filtration rate (GFR), thus producing an increase in urine formation at its source. The increase in GFR is a result of a number of hemodynamic and glomerular mechanisms. ANP dilates the afferent arteriole whilst increasing resistence in the efferent arteriole, thus raising the hydraulic pressure in the glomerular capillaries, a condition which favors urine formation (Zeidel and Brenner, 1987; Awazu and Ichikawa, 1993). In addition, the glomerular filtration surface is increased by relaxation of the mesangial cells (Brenner et al., 1990).

Natriuretic peptides also have post-glomerular affects on the kidney tubules. Some researchers initially proposed that the increase in natriuresis could be accounted for by the stimulation of GFR alone However, such an explanation is now unlikely because the natriuresis is so large, can be stimulated by ANP in preparations maintaining relatively low GFRs, and also because NPs exert secondary affects on kidney function. It is now clear that in addition to increasing GFR, ANP directly inhibits sodium reabsorption in the inner medullary collecting duct (IMCD) and directly and indirectly prohibits water reabsorption by altering hydraulic pressure gradients and inhibiting ADH (Zeidel and Brenner, 1987;

Brenner et al., 1990). ANP directly inhibits amiloride sensitive sodium channels on the luminal surface of IMCD cells, thus preventing entry of sodium into these cells. In addition, there appears to be an ANP stimulated secretory flux via the Na^+/K^+-$2Cl^-$ co-transporter located on the basolateral side (Brenner et al., 1990; Awazu and Ichikawa, 1993). The NP-induced inhibition of sodium reabsorption in the collecting duct appears to be also partly due to altered hydraulic gradients favoring sodium excretion and to washout of medullary solute gradients because of increased vasa recta blood flow (Brenner et al., 1990). ANP indirectly affects sodium transport by inhibiting the renin-angiotensin system: the release of renin from the juxtaglomerular apparatus, and aldosterone from the adrenal cortex, is inhibited. Angiotensin II mediated sodium and water reabsorption is also inhibited in the proximal tubule (Brenner et al., 1990; Awazu and Ichikawa, 1993).

Natriuretic peptide receptors in the various portions of the kidney have been well characterized using radioligand binding assays, and competition and displacement studies, similar to the techniques used in Chapter 2 above (Anand-Srivastava and Trachte, 1993). Early receptor studies demonstrated that ANP binding was located in the glomeruli, the ascending limb of the loop of Henle, and in the collecting ducts but not in the proximal tubule; later studies showed binding in the proximal tubule also; the majority of receptors found in the kidney are either NPR-A or NPR-C (Anand-Srivastava and Trachte, 1993). A study using reverse transcriptase-polymerase chain reaction techniques showed that NPR-A mRNA occurs in the rat glomerulus and throughout the kidney tubule (Terada et al., 1991). Eighty percent of NPRs in the rat glomerulus are of the NPR-C type; however in the papillary regions of the kidney only those of the NPR-A type are found (Martin et al., 1989). Most studies confirm the predominence of NPR-A in the collecting ducts and papillary regions of the kidney and the predominence of NPR-C in the glomeruli and cortical regions (Anand-Srivastava and Trachte, 1993). Recently NPR-B mRNA has been located by polymerase chain reaction in the human kidney suggesting a role for CNP in the kidney (Canaan-Kuhl et al., 1992), which may well be the case since CNP induces

natriuresis in the rat (Sudoh et al., 1990), but antinatriuresis in the dog; a finding which suggests some renal affects may be species specific (Stingo et al., 1992).

The signal transduction mechanisms of NPRs in the kidney have been extensively studied. ANP appears to produce natriuresis via the NPR-A stimulation of cGMP production (Gunning et al., 1989; Anand-Srivastava and Trachte, 1993). ANP also appears to reduce cyclic AMP concentrations in a variety of kidney regions such as the glomeruli, collecting duct, and loop of Henle (Arnand-Srivastava et al., 1986). The adenyl cyclase/cAMP second messenger pathway has been shown in some studies to affect GFR (Dousa et al., 1980). NPR-C has been implicated in inhibiting cAMP pathways; it is also abundant in the glomerulus: both of these findings suggest that some of the effect of NPs on GFR may be mediated via NPR-C (Arnand-Srivastava, 1992; Anand-Srivastava and Trachte, 1993). However, C-ANF, the NPR-C specific NP analogue, failed to produce a diuretic effect in rat kidneys (Maack et al., 1987).

In recent years, a kidney specific NP has been isolated from human urine and named urodilatin (Schultz-Knappe et al., 1988). This peptide has the same structure as ANP but has an additional 4 residues on the NH_2 terminus. It is unknown whether urodilatin is the product of a separate NP gene or is an alternative post-translational product of the ANP gene (Goetz, 1991; Abassi et al., 1992). However, it appears likely that urodilatin is a post-translational product of the ANP gene because the additional 4 amino acids are the same as those that immediately precede the ANP sequence in the prohormone (Abassi et al., 1992), and because renal expression of the ANP gene has been demonstrated in the distal cortical nephrons of intact rat kidneys and in rat distal cortical tubular epithelial cell cultures (Greenwald et al., 1992).

Urodilatin binds to the same kidney receptor sites as ANP in the kidney, and stimulates cGMP production to the same extent, implying the use of the same receptors for both peptides (Valentin et al., 1993). ANP and urodilatin are processed differently by neutral endopeptidase EC 3.4.24.11 (NEP) present on the luminal brush border of the

proximal tubule. ANP is filtered into the lumen of the kidney tubule where much of it is degraded by NEP; urodilatin, which does not circulate, appears to be quite resistent to NEP, an affect which is hypothesized to result in increased delivery of urodilatin to distal portions of the tubule (Goetz, 1991; Abassi et al., 1992). Such a system suggests the presence of NPRs on the luminal border of the distal tubule and collecting duct, but as yet there is no direct evidence for such a placement of receptors. One study, nevertheless, strongly suggests the presence of NPRs on the luminal side of collecting duct cells. Sonnenberg (1990) microperfused the IMCD and compared Na^+ efflux in control preparations with that obtained for ANP or amiloride perfused preparations. Both ANP and amiloride inhibited transport of Na^+ across the duct luminal membrane. Recently, it has been postulated that, while ANP is responsible for the cardiovascular affects, urodilatin rather than ANP is the primary kidney natriuretic hormone (Goetz, 1991; Abassi et al., 1992, Goetz, 1993).

Natriuretic Peptides and the Fish Kidney

Unlike mammals, in which the chief site of salt and water regulation is the kidney, fish partition their osmoregulatory mechanisms between the gills (and the elasmobranch rectal gland) and the kidney. Kidneys are the major site of water regulation in fish with the gills (or rectal gland) being the major salt regulating tissue (see Evans, 1993 for review). The morphology of fish kidneys vary according to their class and environment. Most fishes have glomeruli similar to those of mammals, but, there are some aglomerular species among advanced marine teleosts. This condition probably evolved because high urine output is not a desired characteristic in animals that need to conserve water. Aglomerular fishes have a simple secretory kidney tubule that corresponds to the proximal tubule (segment II) of other teleosts and elasmobranchs. The glomerular kidneys of teleosts are simple in comparison to the kidney of elasmobranchs, some have only proximal tubules, whereas others include a short distal segment before the collecting duct. Elasmobranchs

have a highly complex kidney tubule with all segments present, including an intermediate segment between the proximal and distal tubules, presumably a mechanism for urea retention, an osmoregulatory strategy in this class.

The Agnathans, the lampreys and hagfishes, have different types of kidney. The lamprey kidney is quite elaborate with both glomeruli and all tubule segments present (Hentschel and Elger, 1989). The paired hagfish kidney, including that of Myxine glutinosa, is a persistent mesonephros with very large glomeruli (0.7 - 1.0 mm in length) arranged segmentally along the body, caudal to the heart (Figure 3-1). The glomeruli are paired in each muscle segment on either side of the dorsal midline and each glomerulus is drained by a short neck segment into an archinephric duct (AND) which traverses the length of the body and drains into the cloaca. The morphology of the glomerulus is similar to that of mammals, being perfused from a single afferent arteriole and drained by paired efferent arterioles. In spite of their large size there is some question whether the glomeruli are capable of filtering the blood at normal hagfish blood pressures. It has been postulated that the glomeruli may be of an on/off type, operational during times of increased blood pressure or volume loading, when urine is filtered into the tubule. During times of lower blood pressure, the kidney would function as a secretory type, when urine is formed by secretion of solutes and the subsequent passive movement of water into the duct,. The neck segments and AND are functionally and structurally similar to proximal tubules. They are lined by epithelial cells with an extensive luminal brush border and appear to operate in divalent ion regulation and the reabsorption of glucose and amino acids. Neither salt nor water appear to be reabsorbed, so that the GFR is at unity with the urine flow rate (Munz and McFarland, 1964; Alt et al., 1981; Fels et al., 1989; Evans, 1993).

Because of the renal effects of NPs in mammals, a number of studies have examined renal effects of NPs in fish. Earlier studies focussed on bolus injections or continuous infusions of heterologous ANP, or conspecific heart extracts, into cannulated fishes, followed by the measurement of renal responses (Duff and Olson, 1986; Lee and

Malvin, 1987; Benyajati and Yokata, 1990). In both the glomerular rainbow trout, Salmo gairdneri (Duff and Olson, 1986) and the aglomerular toadfish, Opsanus tau (Lee and Malvin, 1987) ANP produced a significant natriuresis, a significant diuresis in the toadfish, but only a mild diuresis in the trout. In the trout K^+ excretion was also increased, whereas K^+, Mg^{2+}, and Ca^{2+}, were unaltered in the toadfish. In the dogfish shark, Squalus acanthias, heterologous ANP produced a decrease in GFR and a decrease in electrolyte excretion in direct contrast to the findings in mammals, the trout, and the toadfish (Benyajati and Yokota, 1990). In a recent study using homologous ANP and VNP in the eel, Anguilla japonica, Takei and Balment (1993) found that these peptides caused a marked reduction in urine flow rate in freshwater eels but did not alter Na^+ excretion. A comparison of the effects of eel ANP with rat ANP in the trout kidney indicated a similar but slightly less potent natriuresis and diuresis by eel ANP. Eel ANP did not increase K^+ excretion, unlike rat ANP in the previous study (Olson and Duff, 1992).

Perhaps because of the somewhat contradictory results from these studies, more recent research has concentrated on NPR and second messenger dynamics in the fish kidney. ANP binding sites have been autoradiographically observed in the aglomerular kidney of two species of antarctic icefish (Uva et al., 1993) and in the kidney of Myxine glutinosa (Kloas et al., 1988). In the antarctic fishes, ANP specific binding was observed on the basal layer of the tubules and also in the apical brush border of the epithelial cells (Uva et al., 1993). In the glomerular hagfish kidney, the majority ANP binds specifically to the glomeruli, particularly to the arterioles, the inner epithelia of the Bowman's capsule, and the neck segment. In the AND specific binding was observed on the smooth muscle layer surrounding the intraluminal cells of the tubules, but not apparently on these cells themselves. In the same study ANP specific binding was located on the ventral aorta, both on the smooth muscle layer and the endothelium (Kloas et al., 1988). Interestingly, no NP binding was observed in the aglomerular kidney of the Gulf toadfish, Opsanus beta

(Donald et al., in press), although Lee and Malvin (1987) have shown renal effects of NPs in the congener Opsanu tau.

A series of studies have utilized the blockade effect of the NPR-C inhibitor SC 46542 to increase the effective concentration of circulating NPs in trout (Duff and Olson, 1992; Olson and Duff, 1993). The first study demonstrated that the infusion of SC 46542 simulated the renal and cardiovascular effects of exogenous ANP found in previous studies. The subsequent study indicated that the gills were capable of clearing ^{125}I-ANP rapidly from the circulation. However, when gill receptors, presumably NPR-C, were blocked with SC 46542, radiolabel was concentrated in other tissues including the kidney. Recently, heterologous ANP has been shown to stimulate cGMP production in the trout nephron; in the same study Japanese eel ANP was found to stimulate cGMP accumulation in the congeneric FW European eel (Perrott et al., 1993).

The present study re-examines ^{125}I-ANP binding sites in the kidney and ventral aorta of Myxine glutinosa. The presence of ^{125}I-ANP binding sites on the dorsal aorta was also determined since this tissue is dissected from hagfish together with the paired kidneys. As an extension of the study by Kloas et al. (1988), ^{125}I-CNP binding was also examined in these tissues. The relative displacement of iodinated radioligands by non-radioactive rANP, pCNP, and C-ANF was assessed. Competition binding studies, generated from image analysis of autoradiographic data, were performed to determine the relative binding capabilities of rANP, pCNP, and C-ANF to the glomeruli.

Materials and Methods

Autoradiography

Hagfish were collected and maintained as before (Chapter 2). Complete kidneys were removed from hagfish. The ventral aorta was dissected from immediately craniad to the ventricle until the end of the gill arches; part of the afferent branchial arches were left

attached to the aorta. The aortae and short strips of kidney, with the AND, several glomeruli, and the dorsal aorta, intact were then mounted in Tissue Tek. Frozen sections were then prepared and autoradiography and nuclear track emulsion protocols were performed as previously described for the gill tissues, using both ^{125}I-ANP and ^{125}I-CNP as radioligands (Chapter 2).

Competition Binding Assays

Pilot studies indicated that NP binding sites were in insufficient concentrations in membrane preparations to sucessfully perform saturation binding studies, competition binding assays, or affinity cross-linking and SDS-PAGE. An alternative protocol was adopted for competition binding in kidney tissue. Three sets of seven serial sections were prepared. Each slide was comprised of kidney sections from 3 individual hagfish with sections containing at least three different glomeruli for each hagfish.

Sets of sections were preincubated for 15 min at room temperature (22-24 °C) in incubation buffer (Chapter 2). Each set of 7 slides was then assigned for incubation with 200 pM ^{125}I-ANP in the presence of increasing concentrations (10^{-12} - 10^{-6} M) of either rANP, pCNP, or C-ANF added to the incubation buffer to give a visual competition curve. The sections were incubated in the appropriate treatment for 90 min. Following incubation, the slides were processed and exposed to X-Ray film (Chapter 2). Autoradiographs of individual slides were mounted on microscope slides and viewed with an Olympus BH2 light microscope with a X 4 objective. Images of the individual glomeruli were captured on TDK E-HG video film using a Sony DXC-107 video camera with CCD iris and a Sony CMA-D7 camera adapter connected to a Mitsubishi HSU67 video cassette recorder. The glomerular images were then imported onto a Macintosh Quadra and the glomerular area analysed for a mean grayscale value using the NIH Image program (Version 1.54, 1994). A mean for each animal's glomerular grayscale value was computed for every concentration of the competing peptides. The mean ± SE for the 3 animals was then calculated and the %

of maximum value was determined for each data point. Competition binding curves were then generated for each competing peptide.

Guanylate Cyclase Assays

Three kidney membrane preparations were made using 3 hagfish kidneys per preparation. Guanylate cyclase activity was measured for three separate membrane preparations as described above (Chapter 2). The data are presented as the means ± SE of the 3 membrane pools and are plotted as % of basal cGMP production rate. Analyses of variance (ANOVAS) were carried out using the Statview SE program (Abacus Concepts, 1988).

Results

Autoradiography

^{125}I-ANP specific binding was located on both the ventral and dorsal aortae, the AND, and the glomeruli of the kidney (Figs. 3-2A and 3-3A). Specific binding was displaced by 1μM rANP, indicating the level of nonspecific binding; the nonspecific background appeared to be quite high in the ventral aorta (Figs. 3-2B and 3-3B). Specific binding was not displaced to any great extent by 1 μM pCNP or C-ANF (Figs. 3-2C,D and 3-3C,D). There was no specific ^{125}I-CNP binding observed on either tissue (Fig. 3-4A and B). In order to better define the areas of ^{125}I-ANP specific binding in the kidney some slides were dipped in nuclear track emulsion. The areas of particular interest are indicated on the enlargement of the relevant kidney section autoradiograph (Fig. 3-5). Silver grains were densest over the glomeruli (Fig. 3-6A) but the particular cell types were not able to be resolved. Non-specific binding of silver grains is shown in Fig. 3-6B. Silver grains were scattered over both the endothelial and smooth muscle layers of the dorsal aorta (Fig. 3-6C), the majority of silver grains were again displaced by 1 μM rANP (Fig. 3-6D). In the

neck segment leading from the glomerulus to the AND, specific binding was predominently located over the luminal surface of the epithelial cells (arrow, Fig. 3-7A), to the right of the neck segment some glomerular tissue is shown with silver grains scattered over the cells in the same manner observed in Fig. 3-6A. Similar to the distribution in the neck segment, the densest distribution of silver grains appeared along the luminal surface of AND epithelial cells (arrow, Fig. 3-7C). Specific binding in both sections of the duct system was displaced by 1 μM rANP (Figs. 3-7B and D).

Competition Binding Assays

Competition binding analysis was performed using a gray scale image analysis on autoradiographs of glomeruli; Fig. 3-8A-G shows an example of ^{125}I-ANP competing with increasing concentrations of rANP (10^{-12} - 10^{-6} M) for specific binding sites in kidney sections. The intensity of binding diminishes in a dose-dependent manner with increasing concentrations of cold peptide. Competition curves were generated from the gray scale values for each competing cold peptide (rANP, pCNP and C-ANF, Fig. 3-9). Four nanomolar rANP competed for 50 % of ^{125}I-ANP binding sites. Porcine CNP did not compete with specific binding below a concentration of 10 nM; 300 nM pCNP competed for 50 % of the ^{125}I-ANP sites. Porcine CNP displaced all but 20 % of ^{125}I-ANP specific binding at the maximum concentration used in this study (1μM). C-ANF failed to compete for ^{125}I-ANP specific binding sites (Fig. 3-9).

Guanylate Cyclase Assays

Rat ANP significantly stimulated cGMP production 40 % - 50 % above basal rates at concentrations of 10 nM and greater (Fig. 3-10); rANP stimulated cGMP production rate appeared to plateau at these concentrations. Porcine CNP significantly elevated cGMP production 45% - 50 % above basal levels between 0.1 μM and 1 μM; cGMP production

also appeared to be reaching a plateau at these levels. C-ANF failed to stimulate cGMP production above basal levels at any concentration.

Discussion

The specific NP binding profile observed in the kidney and the aortic vasculature of Myxine glutinosa appears very different from that observed in the gills (Chapter 2). In the gill, both ^{125}I-ANP and ^{125}I-CNP specific binding were observed. The binding sites in the gill were resolved into two receptors: the first demonstrated similarities to the mammalian NPR-A, binding ANP preferentially; the second bound all NPs including C-ANF. Only ^{125}I-ANP binding was observed in the kidney and aortae, ^{125}I-CNP did not bind to any tissue (Figs. 3-2 to 3-4). Additionally, pCNP and C-ANF failed to successfully displace ^{125}I-ANP binding (Figs. 3-2 and 3-3). The competition studies were consistent with the autoradiographical results (Fig. 3-9). Rat ANP displaced 50 % of ^{125}I-ANP binding in the glomeruli at a concentration comparable with that found in the gill study (4.0 nM and 1.0 nM, respectively). There was an order of magnitude difference between pCNP 50 % displacement in the gills (20 nM) and the glomeruli (300 nM). C-ANF, which displaced ^{125}I-ANP binding in the gill, failed to displace the radioligand in the kidney. It is clear from these data that the kidney and the aortae lack the 'promiscuous' Site 2 receptor that is present in the hagfish gill.

The stimulation of cGMP production by rANP in kidney membranes was less marked than in gill membranes; rANP significantly stimulated cGMP production above basal levels at concentrations of 10 nM and greater, as opposed to 0.1 nM in the gill (Fig. 3-10). Porcine CNP, similar to its effect in the gill, only stimulated cGMP production at 0.1 μM and 1 μM. However, pCNP stimulated cGMP production to the same extent as rANP at these concentrations, an observation that contrasts with the gill data, in which rANP was the more potent stimulator of cGMP production. C-ANF, as in the gill, failed to produce an increase in the cGMP production rate. The stimulation of cGMP production by

NPs in the kidney may be interpreted as evidence for a GC-linked NPR in this tissue. The less sensitive stimulation of cGMP production in comparison with that found in the gills may be attributed to the preparation of the kidney membranes. The NPR population to protein ratio in these crude pooled preparations is probably quite low; an hypothesis which is reinforced by the failure to produce autoradiograms from affinity cross-linked membranes in this study (see methods section above).

The discrepancies between the GC assays in the gill and kidney notwithstanding, the available data suggest that the predominent receptor type in the kidney and the vasculature is of the NPR-A type (Site 1) originally described in gill tissue. The following reasons are offered as arguments: 1) the binding sites appear particularly sensitive to ^{125}I-ANP; 2) the displacement capability of rANP for this site is similar in both the gill and the glomeruli; 3) there is no observable ^{125}I-CNP binding; pCNP displaces ^{125}I-ANP binding and stimulates cGMP production, but only at concentrations in excess of 0.1 μM (CNP was shown to have a low affinity for this site in the gill); 4) C-ANF does not appear to bind to this site, or stimulate cGMP production, at the concentrations examined.

This study confirms the original findings of Kloas et al. (1988), with the exception that Kloas and coworkers found no discernible binding on the luminal side of the AND epithelial cells, in direct contrast with this study in which the location of the densest duct binding was on the luminal cell border (Fig. 3-7). The majority of binding in the earlier study was found on the smooth muscle layer adjacent to the epithelial layer (Kloas et al., 1988). Although there was some displacable binding observed on the smooth muscle cells in this study (not shown), it was not to the extent observed on the AND and neck segment epithelium. The placement of specific binding along the luminal borders is similar to the binding observed on the apical border of the luminal cells in the kidney of antarctic fishes (Uva et al., 1993). The luminal position of silver grains is also noteworthy in light of the current hypotheses that urodilatin (and ANP) binds to the luminal border of mammalian collecting duct cells (Goetz, 1991).

Clearly, an extensive population of NPRs are present in the glomerulus (Figs. 3-2, 3-5, and 3-6). NPs possibly have some affect on the filtration dynamics in this structure, as has been reported for mammals (Zeidel and Brenner, 1987; Brenner et al., 1990; Awazu and Ichikawa, 1993). However, ANP infusion into a single perfused hagfish glomerulus showed no marked effect (Fels, et al., 1989). Interestingly, the majority of receptors in the mammalian glomerulus are of the NPR-C type, with a smaller population of NPR-A (Martin et al., 1989; Awazu and Ichikawa, 1993), in contrast to the hagfish glomerulus in which the NPR-A-like receptor alone is identified. NPR-A is reported to be the predominent receptor type in the tubular portions of the mammalian nephron (Martin et al., 1989; Awazu and Ichikawa, 1993), a condition which is also apparent in the hagfish, if we assume some homology between the mammalian and hagfish 'A' receptors. It is difficult to hypothesize on the effect of NPs in the AND and neck segment since no salt or water transport has been found in the hagfish kidney (Fels et al., 1989); possibly other transport systems, such as those for acid/base or divalent ion regulation, are affected.

The hagfish ventral and dorsal aortae also appear to be endowed with a major population of the Site 1 receptor. Although no competition or GC assay data exists for these tissues, it is evident from the similarity between the kidney and vascular autoradiographic data that the Site 1 receptor predominates, and that the 'promiscuous' receptor (Site 2), characterized in the gills, is absent (Figs. 3-2, 3-3, and 3-4). Natriuretic peptide receptor populations have been determined in mammalian cultured vascular smooth muscle and vascular endothelial cell lines (Redmond et al., 1990; Fethiere et al., 1992; Suga et al; 1992b). The predominent form of receptor in these cell lines appears to be NPR-C. There are also small populations of GC receptors, but the NPR type depends on the cell line; some expressing NPR-A preferentially, whilst others express the CNP receptor, NPR-B (see Anand-Srivastava and Trachte, 1993 for review). A recent study on NPR types in bovine aortic smooth muscle cells from fresh aortic membrane preparations, and from cell culture, indicated that NPR-C only predominated in the cell culture line, with

the expression of NPR-A disappearing over time. The majority of NPRs in the fresh membrane preparations were NPR-A, suggesting that caution should be used in interpreting the NPR data from cultured cell lines (Abe et al., 1993). The predominence of the NPR-A type receptor in fresh hagfish tissue sections is consistent with the recent discovery of a major NPR-A population in fresh bovine aortic smooth muscle preparations (Abe et al., 1993). The presence of NPRs on the vascular smooth muscle supports the vasodilatory function of NPs, not only in other fishes and mammals, but in the hagfish itself (Evans et al., 1989; Evans, 1991; Evans and Takei, 1992; Evans et al., 1993). The role of NPs in hagfish vascular endothelial function is unknown.

The systemic NPR system in the hagfish tissues examined to date must operate principally via the NPR-A receptor, since it was the only NPR identified outside the gills. Apart from the vasodilatory action of NPs in the hagfish, we have no knowledge of the functions of NPs in the glomerulus, ducts of the kidney, or the vascular endothelium. The hagfish NPR-A appears to be linked with GC activity, and it is presumed, therefore, that any post-gill systemic affects are mediated through the guanylate cyclase/cGMP second messenger system.

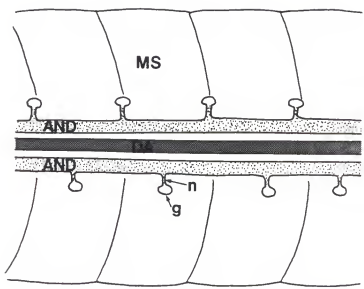

Figure 3-1. Diagram of the position of kidneys and dorsal aorta in the hagfish trunk, indicating major structures.
AND, archinephric duct; MS, muscle segment; DA, dorsal aorta; n, neck segment; g, glomerulus.

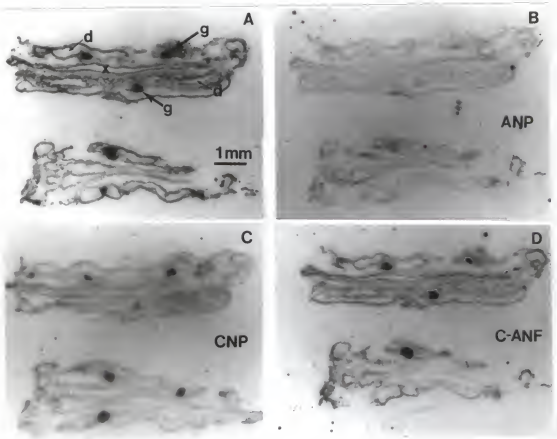

Figure 3-2. Autoradiographs of ^{125}I-ANP binding in serial longitudinal sections through both hagfish kidneys and dorsal aorta.
A. ^{125}I-ANP binding to glomeruli (large arrows, g), archinephric ducts (small arrows, d), and dorsal aorta (x).
B. ^{125}I-ANP + 1 μM rANP showing displacement of specific binding.
C. ^{125}I-ANP + 1 μM pCNP showing slight displacement of specific binding.
D. ^{125}I-ANP + 1 μM C-ANF showing lack of visible displacement of specific binding.

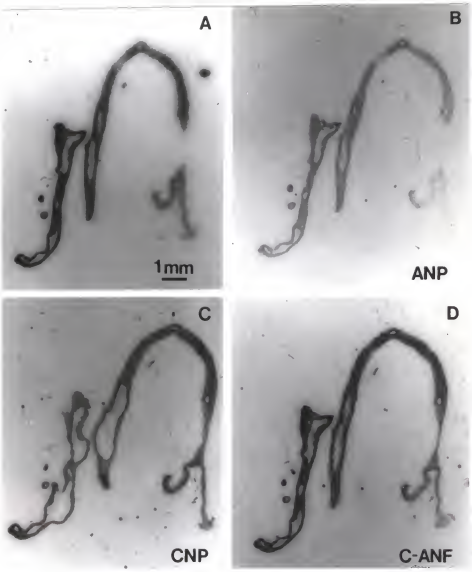

Figure 3-3. Autoradiographs of ^{125}I-ANP binding in serial longitudinal sections through the hagfish ventral aorta.
A. ^{125}I-ANP specific binding.
B. ^{125}I-ANP + 1 μM rANP showing displacement of specific binding.
C. ^{125}I-ANP + 1μM pCNP showing slight displacement of specific binding.
D. ^{125}I-ANP + 1 μM C-ANF showing lack of visible displacement of specific binding.

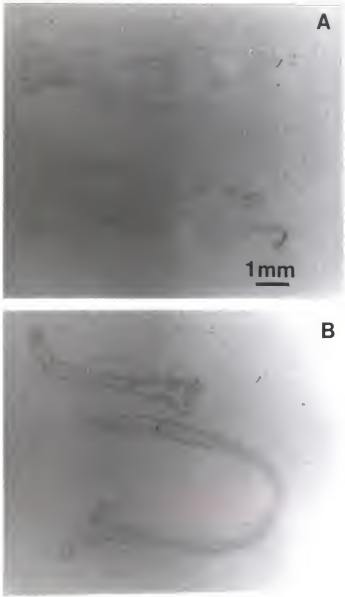

Figure 3-4. Autoradiographs ^{125}I-CNP binding in longitudinal sections through both hagfish kidneys and dorsal aorta (A) and the ventral aorta (B).

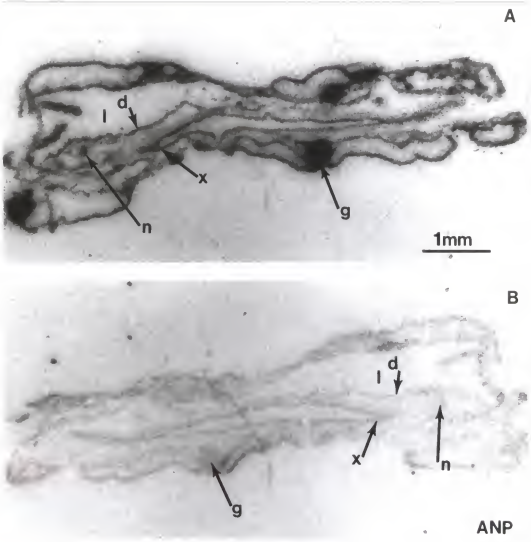

Figure 3-5. Enlargement of autoradiograph of ^{125}I-ANP binding in longitudinal sections through both hagfish kidneys and dorsal aorta, showing specific regions of tissue.
A. ^{125}I-ANP specific binding: g, glomerulus; x, dorsal aorta; d, archinephric duct; n, neck segment.
B. ^{125}I-ANP + 1μM rANP indicating displacement of specific binding. Letters refer to the same structures as in A.

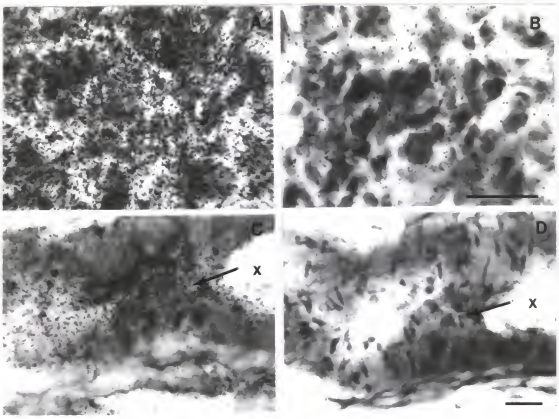

Figure 3-6. Light micrograph of longitudinal sections of glomeruli (A,B) and dorsal aorta (C,D) dipped in X-ray sensitive emulsion showing the distribution of ^{125}I-ANP specific binding. Scale bar A: 13.0 mm = 25 µm. Scale bar B: 11.0 mm = 25µm.
A. ^{125}I-ANP specific binding to the glomerulus. Specific binding is indicated by the density of the silver grains.
B. ^{125}I-ANP + 1 µM rANP showing displacement of specific binding from the glomerulus. Non-specific binding is indicated by the light scattering of silver grains across the glomerular surface.
C. ^{125}I-ANP specific binding to the dorsal aorta (arrow, x)
D. ^{125}I-ANP + 1 µM rANP showing displacement of specific binding from the dorsal aorta.

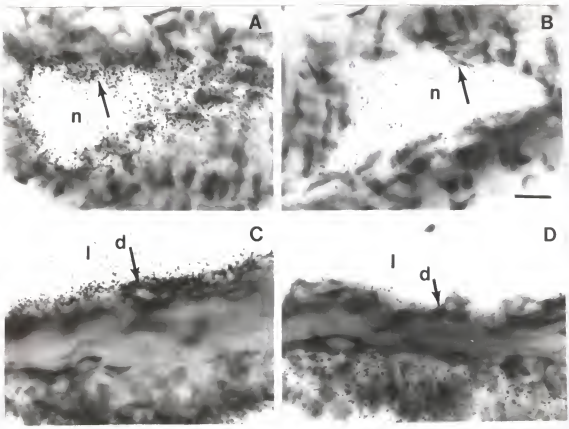

Figure 3-7. Light micrograph of longitudinal sections of neck segment (A,B) and archinephric duct (C,D) of the hagfish kidney dipped in X-ray sensitive emulsion showing the distribution of ^{125}I-ANP specific binding. Scale bar: 10.0 mm = 25 μm.
A. ^{125} I-ANP specific binding to epithelial cells (arrow) of neck segment (n). Specific binding is shown by the density of the silver grains.
B. ^{125} I-ANP + 1 μM rANP showing displacement of specific binding. Non specific binding is indicated by the light scattering of silver grains across the section.
C. ^{125} I-ANP specific binding to the luminal side of the epithelial cells of the archinephric duct.
D. ^{125} I-ANP + 1 μM rANP showing displacement of specific binding.

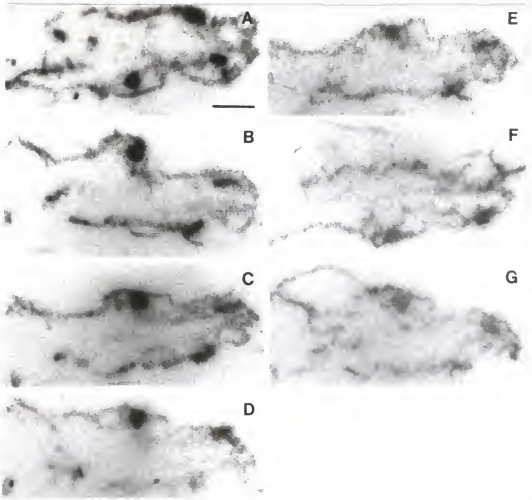

Figure 3-8. Autoradiographs of longitudinal hagfish kidney sections showing displacement of ^{125}I-ANP specific binding at various concentrations of rANP. Scale bar: 1 mm.

A. ^{125}I-ANP + 10^{-12} M rANP.
B. ^{125}I-ANP + 10^{-11} M rANP.
C. ^{125}I-ANP + 10^{-10} M rANP.
D. ^{125}I-ANP + 10^{-9} M rANP.
E. ^{125}I-ANP + 10^{-8} M rANP.
F. ^{125}I-ANP + 10^{-7} M rANP.
G. ^{125}I-ANP + 10^{-6} M rANP.

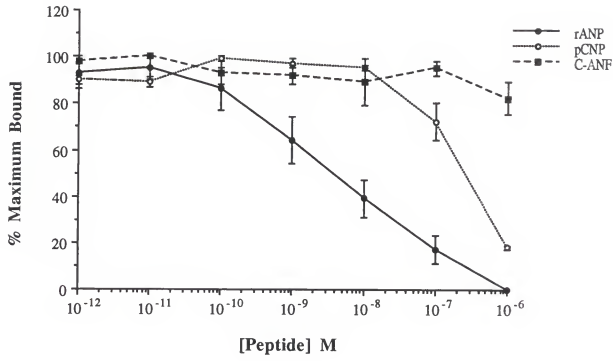

Figure 3-9. Competition study indicating the relative abilities of rANP, pCNP, and C-ANF at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish glomeruli. Each point is the mean ± SE of a mean grayscale value of the autoradiographs of glomeruli from 3 hagfish.

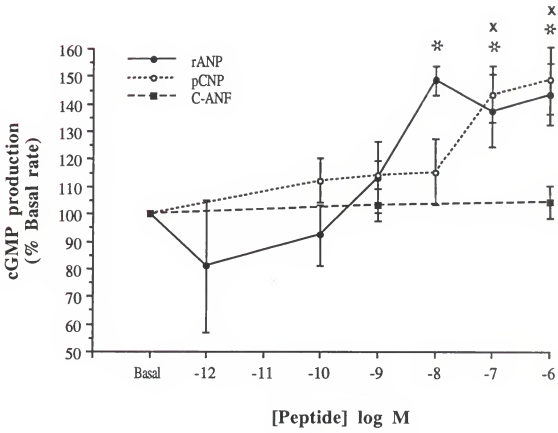

Figure 3-10. Effects of natriuretic peptides on cGMP production rate in hagfish kidney membrane preparations.
Relative effects of increasing concentrations of rANP, pCNP, C-ANF on cGMP production rate. Data points are mean ± SE of 3 pooled membrane preparations each containing kidneys from 3 individual hagfish. ✻ Significant rANP stimulated increase above basal levels; x significant pCNP stimulated increase above basal levels ($p < 0.05$).

WHOLE ANIMAL VOLUME REGULATION, AND NATRIURETIC PEPTIDE RECEPTORS, IN THE ATLANTIC HAGFISH, MYXINE GLUTINOSA, EXPOSED TO 85 % AND 115 % SEA WATER.

Introduction

Volume Regulation in Hagfish

Previous studies have shown that hagfish osmoconform over a narrow range of salinities (for review see Hardisty, 1979). The Atlantic hagfish, Myxine glutinosa, survives successfully in salinities ranging between 57 % and 130 % sea water, providing that the daily concentration change does not exceed 15 mOsm (Cholette et al., 1970). McFarland and Munz (1965) examined the regulation of body weight and serum composition in the Pacific hagfish, Eptatretus stoutii, during 7 days exposure to a range of low and high salinity waters (40 - 122 % sea water, SW) followed by a 7 day return to 100 % SW. Hagfish in 80 % and 122 % SW survived the salinity perturbations with the maximum weight change occurring on the first day. There was a slow readjustment of body volume over 7 days in hyposaline animals, but no volume readjustment occurred in hypersaline fish. Plasma concentrations were isosmotic with the environment.

The osmotic strategies of the hagfish appear to be very similar to marine invertebrates (Robertson, 1963; Hardisty, 1979). The plasma is nearly isosmotic with the environmental media and comprises largely of the inorganic ions: Na^+, Ca^{2+}, Mg^{2+}, and SO_4^{2-}; these ions, however, are not in complete equilibrium with SW. Sodium tends to be slightly higher, whereas the other components tend to exhibit slightly lower concentrations (Morris, 1960; Bellamy and Chester Jones, 1961; Robertson, 1966). Chloride levels appear to vary according to the study; hypo-, hyper-, and iso-tonicity with reference to the

environment being found (McFarland and Munz, 1958). Intracellular osmolality, however, is lower in its concentration of inorganic ions compared to the blood; the osmotic difference between the extracellular and intracellular compartments is balanced with amino acids and trimethylamine oxide (Robertson, 1966; Cholette et al., 1970). The amino acid pool appears to be implicated in the regulation of cell volume, just as it is in invertebrates (Cholette et al., 1970; Oglesby, 1981).

No volume regulation study on whole animals, similar to that of McFarland and Munz (1965) in which animals are exposed to a sudden salinity change, has been performed with Myxine glutinosa. As a prelude to studies on changes in natriuretic peptide receptor populations in different salinities, we investigated the ability of Myxine glutinosa to survive sudden hypo- and hypersaline stress and its capability, if any, of regulating whole body volume. Changes in hematocrits and blood osmolality were also measured.

Natriuretic Peptides and Volume and Salt Loading in Mammals

Both high salt diet and volume loading tend to produce an increase in circulating levels of NPs in mammals, whereas low salt diets have been shown to decrease circulating NP levels and increase atrial NP storage granules (Brenner et al., 1990; Ruskoaho, 1992; Anand-Srivastava and Trachte, 1993). These changes in circulating NP concentration have been associated with the reciprocal regulation of NPRs (Anand-Srivastava and Trachte, 1993). Dehydration or a low salt diet increase the density of NPRs in rat glomerular membranes, but the opposite is found for rats fed high salt diets (Ballerman et al., 1985; Gauquelin et al., 1988; Kollenda et al., 1990). It is apparent that changes in receptor density tend to be due to the up- or down-regulation of NPR-C; NPR-C populations vary inversely with NP immunoreactivity in the blood (Kollenda et al., 1990). NaCl-treated vascular endothelial cells in culture have shown a similar inverse relationship of NPRs to high salt conditions (Katafuchi et al., 1992). Katafuchi and coworkers demonstrated that NPR-C was downregulated in NaCl treated cells, and there was also an increase in cGMP

production, presumably mediated by NPR-A. Some studies have shown, however, that exposure of cultured cells to NPs not only reduce receptor number but also reduce NP stimulated cGMP production, suggesting a greater complexity of receptor interactions than would be accounted for by the simple clearance function of NPR-C alone (Cahill et al., 1990; Kato et al., 1991). In spite of the complexities of NPR regulation, it is clear that mammalian receptor populations are responsive to the circulating concentration of NPs.

Natriuretic Peptides and the Environmental Salinity of Fish

The discovery of the role of NPs in mammalian salt and water balance was the initial impetus for fish physiologists to search for the presence of NPs in fishes, and a possible role of NPs in fish osmoregulation (Evans, 1990; Evans and Takei, 1992; Evans, in press).

Plasma concentrations of NPs, measured by heterologous radioimmunoassay (NP_{ir}), tend to be higher in fishes from high salinity environments. NP_{ir} was lower in the plasma of the euryhaline teleost, Gila atraria, from a fresh water spring as opposed to the plasma NP_{ir} from the same species collected from a 1 % NaCl spring (Westenfelder et al., 1988). The results from this 'natural' experiment were confirmed in laboratory studies in which fish acclimated to freshwater (FW), 1 % NaCl water, or high salt water, demonstrated a range of plasma NP concentrations, with the highest NP_{ir} correlating with the highest salinity water (Westenfelder et al., 1988). Evans and coworkers (1989) demonstrated a similar relationship between salinity and NP_{ir} in longhorn sculpin, Myoxocephalus octodecimspinosus, and winter flounder, Pseudopleuronectes americanus, acclimated to SW and diluted SW (100 mM Cl^-), as did Smith et al. (1991) in the smolts and parr of Atlantic salmon, Salmo salar, abruptly transferred to SW, and in rainbow trout, Oncorhyncus mykiss, fed either a high salt diet or acclimated to SW. The acclimation of the lamprey, Petromyzon marinus, to SW was also accompanied by an increase in plasma NP_{ir} (Freeman and Bernard, 1990). The eel, Anguilla japonica, however, appears to be an

exception since ANP plasma concentrations decline in SW (Takei and Balment, 1993). Such results suggest that either the eel is an atypical teleost in terms of NP regulation, or that the use of homologous antibodies to native fish peptides, as in the Takei amd Balment study, are necessary to demonstrate realistic physiological effects.

In a study of several species of endemic FW or SW species, atrial and ventricular NP_{ir} and storage granule population were greater in FW than in SW species; however, there was no difference in circulating NP concentrations (Uemura et al., 1990). The density of NP-like storage granules in the hearts of FW adapted eels was also significantly elevated above that observed in SW adapted animals (Broadhead et al., 1992). Radioligand NP binding demonstrated two receptor sites in isolated gill membranes from the SW eel, whereas only a single binding site was resolved for FW eel gills; in addition, SW gills appeared to have a larger NPR population than FW gills, and NP stimulated cGMP production was greater in SW eels (Broadhead et al., 1992).

These studies suggest that a high salt environment, rather than volume loading, may be the stimulus for NP release in teleosts. The results correlate well with those from mammalian studies: decreased NP storage in the heart is associated with increased plasma NP_{ir} in animals exposed to high salt environments or diets. However, Broadhead et al. (1992) found an up-regulation of receptors under SW conditions in the eel, in contrast with NPR down-regulation associated with higher NP plasma concentrations in mammals fed a high salt diet. Nevertheless, the observation by Broadhead et al. (1992) in the eel may not be at odds with mammalian research because Takei and Balment (1993) found a decreased NP_{ir} in SW rather than FW eels. The present study examines the response of Myxine glutinosa NPRs to volume perturbations caused by alterations in the environmental salinity. The salinity receptor research was preceded by a study of volume regulation in hagfish during two weeks of low or high salinity exposure.

Materials and Methods

Volume Regulation and Tissue Preparation

Hagfish were collected and maintained prior to the experiment as previously described (Chapter 2). Fifteen hagfish (mean mass: 40.8 ± 2.5 g) were placed in tared plastic tubs (approximately 1 liter) with drainage holes drilled around the lid and base. Hagfish were then submerged in 100 % SW (927 ± 4 mOsm, n = 6) and allowed to recover from handling. During the next 24 - 48 h each hagfish within its tub was weighed until we were confident that reproducible weights (within 1 %) could be obtained. These weights were averaged to give the pre-experimental weight. Low salinity water (85 % SW) was mixed by diluting SW with distilled water. High salinity water (115 % SW) was prepared by mixing normal SW with Instant Ocean (5.52 g/ L SW). Previous observations had indicated that these salinities were the maximum and minimum comfortably tolerated by the hagfish. Five hagfish were then transferred into each salinity. The remaining five hagfish were maintained in 100 % SW as controls. All hagfish were maintained between 12 - 14 °C. Hagfish were weighed at 6, 12, 24, and 36 h, after the start of the experiment and daily thereafter for 13 days. Percent weight gain or loss were calculated.

Once the time of maximum weight change had been established, a further set of animals was set up in each salinity for sacrifice at this time (12 h for 85 % SW and 3 days for 115 % SW). The hagfish were anaesthetised in MS 222 (1:1000, Sigma, St.Louis, MO) before blood and tissue collection. Blood samples were collected from the caudal sinus and centrifuged in microfuge tubes for osmolality measurements. Blood samples were also collected in heparinized microcapillary tubes and centrifuged in an IEC centrifuge for hematocrit measurement. Plasma and environmental water osmolalities were measured on a Wescor osmometer (Model 5100B). Gill and kidney tissue was also collected. Gills were snap frozen in liquid nitrogen, and stored at -70 °C before isolated gill membranes were prepared for each animal according to the usual protocol (Chapter 2). Kidney tissue

was mounted in Tissue Tek and frozen; kidneys were then sectioned according to the protocol described in Chapters 2 and 3.

Data were analyzed using Statview 512+ (Abacus Concepts Inc. 1988). Paired t-tests were performed at the apha < 0.05 significance level to compare weights of individuals each day with their initial weight before treatment. Osmolalities and hematocrits were analyzed using Student's t-test ($p < 0.05$); the hematocrit data was first transformed to the the arcsin of the square root before analysis.

Competition Binding Assays

Competition binding studies for ^{125}I-ANP specific sites were performed for each experimental group using either isolated gill membrane preparations, or autoradiography of kidney sections followed by image analysis of the glomerular gray scale values. The protocols followed were the same as described in Chapters 2 and 3. Experimental groups were compared to the control data already discussed in Chapters 2 and 3 above. ANOVAs ($p < 0.05$; Statview 512+, Abacus Concepts Inc. 1988) were used to determine significant differences between the experimental groups and the control groups at each concentration of competing peptide. Data were transformed to the arcsin of the square root before analysis.

Results

Volume Regulation, Plasma Osmolality, and Hematocrits

The weight of hagfish in 100 % SW did not differ statistically from the initial weights during the course of the experiment (Fig. 4-1). Hagfish in 85 % SW gained weight rapidly to a maximum of 8 % at 6 h after the initial transfer. Their weight decreased towards normal levels (2 - 4 %) by 36 h. Weights returned to original levels on the second day, although they were not statistically the same on days 3, 6 and 7. Hagfish

in 115 % SW decreased in weight with maximum weight loss (-10 to -8 %) occurring by the end of the first day. By day 4 there was a slight readjustment towards normal levels (-6 to -8 %) but weights were always significantly different from the initial weights. Plasma osmolality and hematocrits were measured at 12 h for 85 % SW hagfish and 3 days for the 115 % SW group, since these were the times immediately preceeding volume compensation. The plasma osmolalities for all three groups were statistically the same as the environmental water for that particular group. Mean values for the environmental water were: 100 % SW, 927 ± 4 mOsm (n = 6); 85 % SW, 788 ± 4 mOsm (n = 3); 115 % SW, 1064 ± 3 mOsm (n = 6). Mean values for hagfish plasma osmolality were: 100 % SW, 927 ± 5 mOsm (n = 9); 85 % SW, 806 ± 46 mOsm (n = 10); 115 % SW, 1049 ± 8 mOsm (n = 9). Hematocrits for all groups were significantly different from each other. Mean hematocrits were: 100 % SW, mean = 28 ± 1 (n = 13); 85 % SW, mean = 21 ± 1 (n = 11); 115 % SW, mean = 31 ± 1 (n = 17).

Competition Binding Studies

Gill membranes

Fifty percent of ^{125}I-ANP specific binding was displaced by 1 nM rANP in control animals as already reported (Chapter 2); rANP concentrations of 0.6 nM and 5 nM, for 115 % SW and 85 % SW respectively, competed for 50 % of the sites. High salinity membranes were significantly different from control values at 0.1 nM and 3 nM; low salinity membranes were different from control values at 1 nM (Figure 4-2). Twenty nanomolar pCNP competed for 50 % of ^{125}I-ANP specific sites in control animals, but 0.9 nM and 2 nM pCNP competed for 50 % of sites in 115 % and 85 % animals. High salinity membranes were significantly different from control values between 0.3 nM and 30 nM; low salinity membranes were different from control values at these concentrations also, with the exception of the value at 10 nM which was not significantly different (Fig. 4-3). There was no difference in C-ANF displacement of ^{125}I-ANP binding sites in hagfish from

any treatment; C-ANF concentrations competing for 50 % of the specific sites were between 15 and 30 nM (Fig. 4-4).

Kidney glomerular sections

Competition for 50 % of ^{125}I-ANP binding sites by rANP in hagfish glomeruli ranged between 1 nM for hagfish in 115 % SW to 4 nM for hagfish in 100 % SW; 3 nM rANP competed for 50 % of sites in hagfish in 85 % SW. Both high and low salinity glomeruli were significantly different from control values at 10 nM, but only 115 % SW glomeruli were different at 100 nM; the remaining values were not different from the control treatments (Fig. 4-5). Three hundred nanomolar pCNP competed for 50 % of the specific sites in control animals; however, 1 nM and 20 nM (115 % SW and 85 % SW, respectively) competed for 50 % of the sites. High salinity glomeruli were different from control glomeruli at 1 and 10 nM; low salinity glomeruli were different from controls at 10 nM and 100 nM (Fig. 4-6). C-ANF did not significantly displace any ^{125}I-ANP specific binding in any of the treatments (Fig. 4-7).

Discussion

Myxine glutinosa is able to compensate for the volume load that occurs after low salinity transfer, but is apparently unable to compensate for the volume decrease resulting from hypersaline exposure (Fig. 4-1). This finding is similar to that found for Eptatretus stoutii, which increased weight by a maximum of 10 % in 80 % SW, but with maximum weight gain occurring less rapidly than in the present study, being reached at 24 h after the initial transfer (McFarland and Munz, 1965). Eptatretus stoutii exposed to 80 % SW approached normal weights at 5 days, indicating that compensation in this species, at this slightly lower salinity, appears to take longer than in the present study. Eptatretus stoutii exposed to 122 % SW lost approximately 15 % of its body weight that was not compensated during the seven days of the experiment. The higher percentage weight changes for Eptatretus stoutii are probably partly due to the slightly more extreme salinities

to which these hagfish were exposed. We also confirm that hagfish plasma becomes isosmotic with the environmental water after transfer to different salinities. The hematocrits conform with the expected trend due to an increase or decrease in blood volume: the higher hematocrit being associated with dehydration in high salinity SW, and the opposite being true for the volume expanded condition of hagfish in low salinity SW. In its responses to high and low salinity stress, Myxine glutinosa, together with Eptatretus stouti, are similar to marine invertebrate osmoconformers, which counteract the effects of a water load/salt loss in low salinities more effectively than they compensate for the salt load/ water loss in high salinity water (Oglesby, 1981).

The results of the radioligand binding assays clearly indicate that the changes in environmental salinity and consequent volume perturbations in the hagfish had no effect on C-ANF competitive binding in either the gills or the glomeruli (Figs. 4-4 and 4-7). The competition of rANP for ^{125}I-ANP binding sites showed a modest, though usually non-significant, adjustment in salinity altered animals, displaying a trend to compete with greater effectiveness in the gills and glomeruli from hagfish exposed to 115 % SW, and with slightly less effectiveness in tissues from hagfish exposed to 85 % SW; although the glomeruli from 85 % SW treated animals demonstrated an increased sensitivity compared with control values at higher concentrations of rANP (Figs. 4-2 and 4-5). The competition of rANP for ^{125}I-ANP binding sites at different salinities in the gill, although largely non-significant compared with control values, suggests a pattern similar to that observed in the eel (Broadhead et al., 1992), in which NP receptors appeared to be fewer in FW eels than in SW eels. Natriuretic peptide receptors appear to be regulated reciprocally by circulating NP plasma concentrations in mammals (Anand-Srivastava and Trachte, 1993); however, we have no plasma NP_{ir} data from this study with which to ascertain whether the same inverse relationship of receptors to circulating NPs exists in the hagfish. Nevertheless, the regulation of peptide hormone receptors by their homologous peptide is a common feature of peptide hormone-receptor interactions (Baxter and Turtle, 1985; Hubbard, 1987), and

consequently, if we extrapolate from the present study on salinity adjusted hagfish, an increase in plasma NP_{ir} in FW, and a decrease in SW, become possibilities to consider.

The greatest change from control values, however, appear in the competition of pCNP for ^{125}I-ANP specific binding sites (Figs. 4-3 and 4-6). The pCNP competition for specific binding sites was far more sensitive in tissues from hagfish from both high and low salinities than in control animals; for instance, in the gill, 115 % SW tissues competed for 50 % of binding site at a concentration of 0.9 nM, and 85 % SW tissues at 2 nM as opposed to 20 nM in the control gills. In the glomeruli, 115 % SW animals showed the highest sensitivity competing for 50 % of binding sites at 1 nM, 20 nM pCNP competed for 50 % of sites in 85 % SW hagfish, whereas 300 nM pCNP competed for 50 % of specific binding in the control. However, the kidney data was less significantly different from control values than was observed in the gill membranes for pCNP competition; this is probably due in part to the small sample size and high varialbility. The increased sensitivity of pCNP to ^{125}I-ANP specific binding sites was displayed at all concentrations between 100 pM and 0.1 µM, where pCNP in the control group displayed a similar competitive capacity to that in the experimental groups.

It is difficult to interpret the somewhat surprising increase in sensitivity of pCNP for hagfish NPRs during salinity stress. Paradoxically, the competition of pCNP for ^{125}I-ANP specific sites was similar in both high and low salinities, which makes both the physiological interpretation of such data, and the reconciliation of the rANP and pCNP results, problematical. It appears that the mechanism for this increased sensitivity, and the more modest alterations in rANP sensitivity, do not involve the 'promiscuous' Site 2 receptor (Chapter 2) since C-ANF competition binding did not change from control binding in either the gill or the glomerulus of salinity adjusted animals. The alteration in pCNP sensitivity appears to be disjunct from the alteration in rANP sensitivity for two reasons: firstly, rANP competitive binding is less sensitive in the gills of 85 % SW adjusted animals than in control animals, whereas pCNP competition is more sensitive in the same 85 % SW

gills compared to controls; secondly, rANP competition in the glomeruli of 85 % SW hagfish is only greater than in the control group at concentrations in excess of 3 nM, whereas the same glomeruli are more susceptible to pCNP competition for ^{125}I-ANP sites at all concentrations greater than 100 pM. It is assumed that the NPR type involved is the NPR-A-like, Site 1 (Chapter 2). Because this salinity perturbation study does not include saturation data and analysis, or experiments utilizing ^{125}I-CNP, it is unknown whether alterations in the competition curves involve changes in the Site 1 receptor number, or differential alterations in affinities of rANP and pCNP for Site 1, or both.

However, an alternative hypothesis exists. Because of the increase in the competitive ability of pCNP for NPRs, it is possible that a new population of receptors is being expressed at measurable levels, as opposed to alterations in the already present Site 1 receptor. Such a receptor population would bind CNP in preference to ANP, and thus would resemble the mammalian NPR-B. This hypothesis could be tested on the kidney using saturation analysis of ^{125}I-ANP and ^{125}I-CNP binding, and ^{125}I-CNP competition analysis; the kidney would be the tissue of choice since under normal SW conditions the Site 1 receptor appears to be the only NPR measurably present. The putative presence of an NPR-B type receptor is given some creedence by the eel study of Broadhead et al. (1992) who observed two NPRs in SW adapted eels that differed in their abilities to bind ANP; they suggested that, since the additional receptor found in SW eels had a low affinity for ANP, it might be of an NPR-B type. As yet no NPR-B regulation studies have been performed on mammalian kidneys, since it is only recently that expression of this receptor subtype has been demonstrated in this tissue (Canaan-Kuhl et al., 1992); the majority of mammalian NPR-B is found in the central nervous system (Anand-Srivastava and Trachte, 1993). However, there is good evidence for an NPR-B being a dominant receptor type in at least one fish group, since Gunning et al. (1993) observed an NPR-B type receptor in the dogfish shark rectal gland, and Donald (unpublished observations) has made similar observations in the gill of the same species. Both studies showed a more potent stimulation

of cGMP by CNP than by ANP. An elasmobranch NPR-B should not be surprising since CNP appears to be the major systemic NP in this group (Schofield et al., 1991; Suzuki et al., 1991 and 1992). However the response of hagfish NPR populations to alterations in environmental salinity are interpreted, it is clearly a fertile subject for future research.

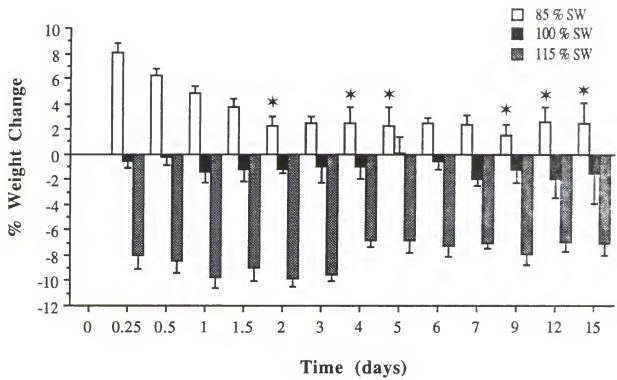

Figure 4-1. Histogram of % weight changes in hagfish exposed to 85 %, 100 %, and 115 % SW during a 15 day period.
*NOT significantly different from initial weights: $p < 0.05$. Weights of hagfish in 100 % SW were not different from initial weights.

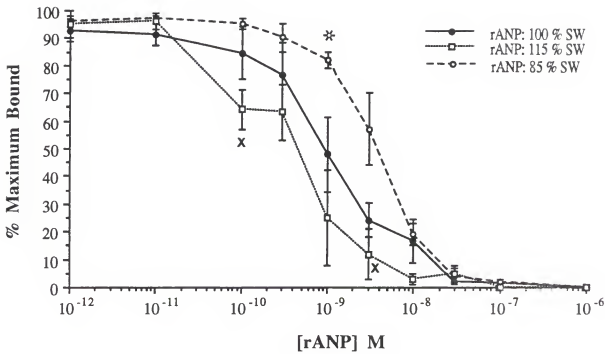

Figure 4-2. Competition study indicating the ability of rANP at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish gills exposed to 85 %, 100 %, and 115 % SW.

25 pM ^{125}I-ANP were added to each membrane incubation reaction. Each point is the mean ± SE of gill membrane preparations from 5 separate hagfish (85 % and 115 % SW); and from 10 hagfish (100 % SW). x 115 % SW group significantly different from control; * 85 % SW group significantly different from control ($p < 0.05$).

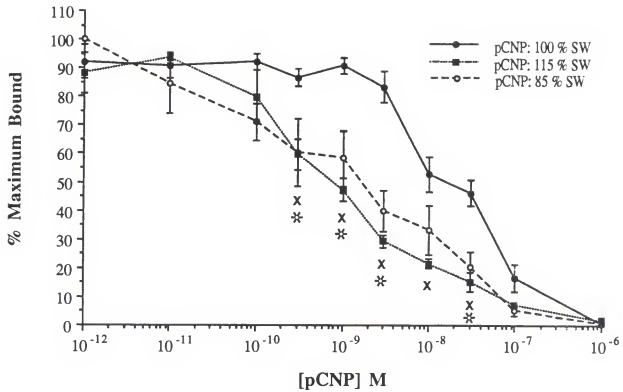

Figure 4-3. Competition study indicating the ability of pCNP at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish gills exposed to 85 %, 100 %, and 115 % SW.
25 pM ^{125}I-ANP were added to each membrane incubation reaction. Each point is the mean ± SE of gill membrane preparations from 5 separate hagfish (85 % and 115 % SW); and from 10 hagfish (100 % SW). x 115 % SW group significantly different from control; * 85 % SW group significantly different from control ($p < 0.05$).

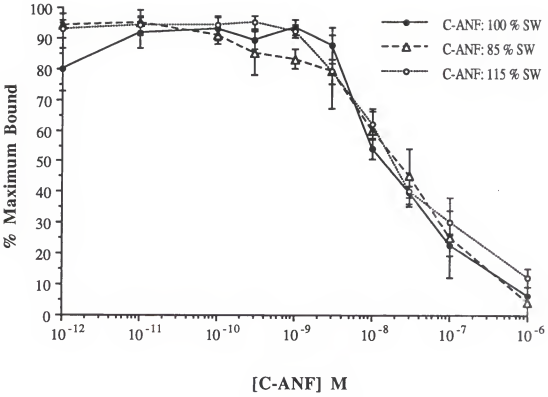

Figure 4-4. Competition study indicating the ability of C-ANF at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish gills exposed to 85 %, 100 %, and 115 % SW.
25 pM ^{125}I-ANP were added to each membrane incubation reaction. Each point is the mean ± SE of gill membrane preparations from 5 separate hagfish (85 % and 115 % SW); and from 10 hagfish (100 % SW).

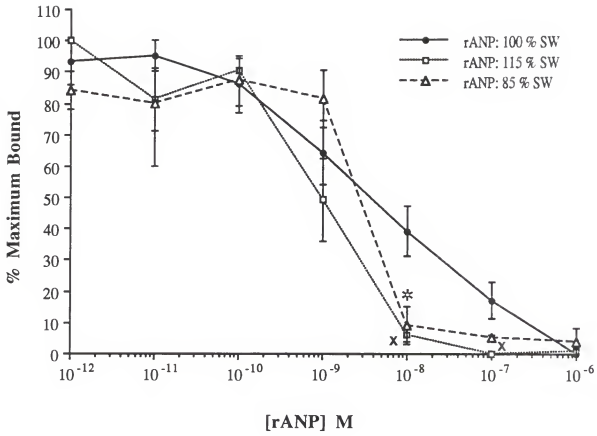

Figure 4-5. Competition study indicating the ability of rANP at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish glomeruli exposed to 85 %, 100 %, and 115 % SW.
Each point is the mean ± SE of a mean grayscale value of the autoradiographs of glomeruli from 3 hagfish. x 115 % SW group significantly different from control; * 85 % SW group significantly different from control ($p < 0.05$).

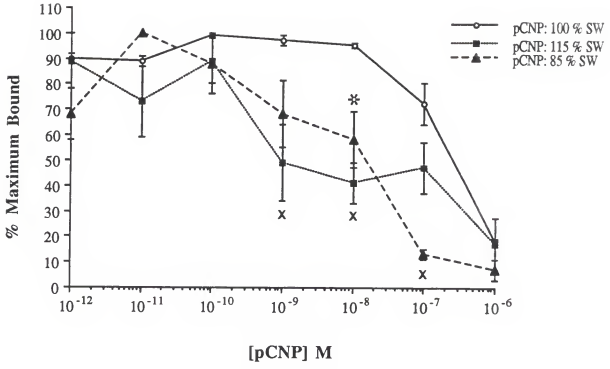

Figure 4-6. Competition study indicating the ability of pCNP at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish glomeruli exposed to 85 %, 100 %, and 115 % SW.
Each point is the mean ± SE of a mean grayscale value of the autoradiographs of glomeruli from 3 hagfish. x 115 % SW group significantly different from control; * 85 % SW group significantly different from control ($p < 0.05$).

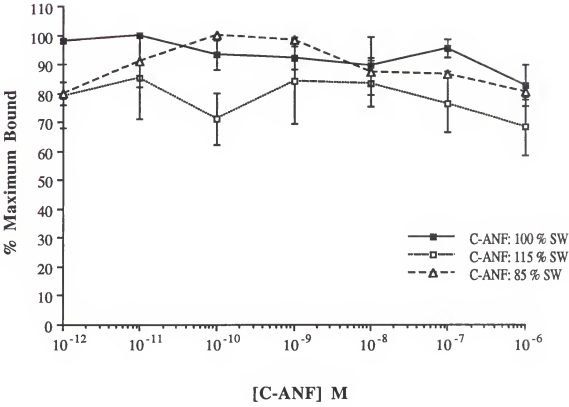

Figure 4-7. Competition study indicating the ability of C-ANF at increasing concentrations to compete for ^{125}I-ANP specific binding sites in hagfish glomeruli exposed to 85 %, 100 %, and 115 % SW.
Each point is the mean ± SE of a mean grayscale value of the autoradiographs of glomeruli from 3 hagfish.

GENERAL DISCUSSION

It is now evident that hagfish have a well-developed NP system. Previous research has shown that not only does the heart, brain, and plasma of Myxine glutinosa have NP-like immunoreactivity (Reinecke et al., 1987; Evans et al., 1989; Donald et al., 1992), but that heterologous NPs are vasoactive, dilating the vascular smooth muscle of the ventral aorta (Evans, 1991; Evans et al., 1993), where binding sites have been located (Kloas et al., 1988; present study). The discovery of NP immumoreactivity and the localization of NP binding sites in the hagfish brain indicate that NPs are neuropeptides, functional in the central nervous system (Donald et al., 1992; Donald and Toop, unpublished). In addition, the presence of binding sites in the glomeruli and archinephric ducts indicates that the hagfish kidney is also a target organ (Kloas et al., 1988; Toop, present study). This study extends NP function to the gills of hagfish, and further elucidates the NPR population in the kidney.

In the gill, the absence of smooth muscle in the lamellar epithelium (Elger, 1987), where NP binding is concentrated, suggests that control of blood flow is not a function of the NPR-A type, Site 1, receptor in this tissue (Chapter 2). However, some mammalian studies have shown potent cGMP stimulation in tissues where binding sites were not observed, presumably because the receptor population was below the detection limit for the assay (Leitman and Murad, 1990). It is possible, therefore, that NP binding could occur on the smooth muscle cells in the other regions of the gill, and thus control local blood flow, without being detected by autoradiography. Nevertheless, NP binding in the lamellar region of the gill must contribute to other functions, such as ion transport or acid/base

regulation. It is probable that the ANP/CNP, Site 2, receptors are concerned, at least in part, with clearance of NPs from the circulation (Chapter 2).

The location of NP receptors on the lamellar epithelium where ionocytes are found invites speculation on the possible effects of NPs on ion flux. Ionocytes are mitochondrion-rich cells similar morphologically to the chloride cells of higher fishes (Elger, 1987). Chloride cells function in salt excretion in marine teleosts (Evans, 1993), an apparently inappropriate function for the osmoconforming hagfish. However, a recent study suggests that salt uptake by FW trout may be facilitated by chloride cells (Perry and Laurent, 1989); branchial salt uptake in FW teleosts is believed to occur via Na^+/H^+ and Cl^-/HCO_3^- exchange, mechanisms that are also believed to be present in the hagfish gill (Evans, 1984). These mechanisms probably developed for acid/base regulation, which, unlike salt regulation, would be valuable for the hagfish that encounters anaerobic environments when burrowing. It has been suggested that the presence of Na^+/H^+ and Cl^-/HCO_3^- exchange mechanisms in the osmoconforming agnathan ancestors of FW fishes were a 'preadaption' for osmoregulation in fresh water (Evans, 1993). Interestingly, there is some evidence that ANP has an inhibitory affect on the Na^+/H^+ antiporter in mammals. In cultured vascular smooth muscle cells, both ANP and cGMP were found to inhibit Na^+ uptake via the Na^+/H^+ antiporter (Caramelo et al., 1994). Sodium ion uptake in the avian intestine was inhibited by ANP and by cGMP (Semrad et al., 1990); the inhibition of Na^+ was accompanied by a decrease in cellular pH, and was not increased by the addition of amiloride (which blocks the antiporter), leading to the conclusion that Na^+/H^+ exchange was compromised in this tissue. It would be interesting, therefore, if NPs, an important hormone in salt and water balance in higher vertebrates, were found to be functional in the control of acid-base regulation in the hagfish gill.

The NP receptor population in the kidney and aortic tissue appears to consist of Site 1, NPR-A type receptors (Chapter 3). In contrast, the gill was observed to have two receptor types (Chapter 2). While NPs are clearly vasodilatory in the hagfish ventral aorta

(Evans, 1991; Evans et al., 1993), and hence presumably function in control of blood flow, we can only speculate as to NP function in the hagfish kidney. The strongest binding in the kidney was associated with the glomerulus (Kloas et al., 1988; Chapter 3), suggesting the hypothesis that NPs affect renal blood flow; however, ANP infusion into a single perfused hagfish glomerulus showed no marked affect (Fels, et al., 1989), although this finding may be attributable to the in vitro nature of the experimental procedure. The presence of NPRs in the archinephric duct and neck segment (Kloas et al., 1988; Chapter 3) are difficult to interpret, since no salt or water transport has been found in the hagfish kidney (Fels et al., 1989). One can only speculate that other transport systems, such as those for acid/base or divalent ion regulation, are affected by NPs. If NPs were involved in acid/base regulation in the gill via modulation of an Na^+/H^+ antiport mechanism, additional NP involvement in acid/base regulation at the kidney would be an appropriate function. So little is known about transport characteristics and function in hagfish that there is a great need for basic research on these parameters before the biology of their control mechanisms can be elucidated. Nevertheless, the presence of NPRs in the hagfish kidney point to an ancient role for the natriuretic peptide family in kidney function.

One of the purposes of this study was to determine whether guanylate cyclase activity was a phylogenetically ancient characteristic of natriuretic peptide receptors. Natriuretic peptides displayed the capacity for stimulating cGMP production in both the hagfish gill and kidney, indicating an ancient coupling of at least one class of NPRs, (probably the Site 1 type in hagfish) to particulate guanylate cyclase (Chapters 2 and 3). What is unknown is whether any second messenger system is associated with the Site 2 gill receptor. The mammalian clearance receptor has been implicated in both cAMP and phosphoinositol pathways (Levin, 1993; Anand-Srivastava and Trachte, 1993); but since the phylogenetic relationship of the homodimeric NPR-C and the Site 2 hagfish gill receptor is unknown, we cannot speculate on possible second messenger pathways mediated by the latter. Nevertheless, if it is subsequently discovered that the same second

messenger pathways are implicated for the hagfish Site 2 NPR as for NPR-C, then a functional homology is implied, which may be interpreted as an indication of convergent evolution if a 'promiscuous' receptor was not present in the hagfish and gnathostome common ancestor. A convergent function may also be inferred if Site 2 shares a similar clearance function with NPR-C. Ultimately, the question of structural similarity between the NPR-C and the hagfish Site 2 NPR must be addressed before a conclusion of convergent function can be assumed, and this question can only be settled by examination of the amino acid sequence of the Site 2 receptor.

Although hagfish are stenohaline osmoconformers, they are obviously capable of volume regulation over a narrow range of salinities (Chapter 4). In fact, Myxine glutinosa has shown itself surprisingly tolerant to quite a range of salinities (57 % - 130 % SW) providing that the salinity change did not exceed 15 mOsm per day (Cholette et al., 1970). Presumably the original influx of vertebrates into estuarine and FW habitats would have occurred over millenia allowing gradual changes in their osmoregulatory physiology. Under such a scenario, preadaptions to life in FW could be selected and refined. Since Na^+/H^+ exchange is a mechanism for sodium uptake in FW fishes (Evans, 1993), and if ANP (and/or cGMP) inhibits sodium uptake via this mechanism in fishes, then it would be in the advantageous for fishes migrating into low salinities, or indigenous FW fishes, to reduce the impact of NPs on Na^+/H^+ exchange in the gill. This could be achieved either by down-regulating GC-coupled receptors, up-regulating non-GC-linked receptors (such as NPR-C or the hagfish Site 2), or by decreasing circulating concentrations of NPs. There have been reports of down-regulation of NPR-C under high salt conditions in mammals, associated with an increase in plasma ANP and an increase in cGMP production; a strategy that would work in mammals when they need to inhibit salt uptake at times of high salt load (Anand-Srivastava and Trachte, 1993).

Unfortunately, receptor data and plasma NP_{ir} data have not been collected for the same fish species during salinity perturbations, with the exception of the eel (Broadhead et

al., 1992; Takei and Balment, 1993). The existing data are suggestive, plasma NP_{ir} is generally lower in fishes acclimated to FW rather than SW (Evans, 1990; Evans and Takei, 1992; Evans, in press). However, the FW eel has a higher plasma NP_{ir} but a smaller NPR population (Broadhead et al., 1992; Takei and Balment, 1993), which would reduce the impact of ANP on the Na^+/H^+ antiporter if NPRs are present in insufficient quantities to mediate the effect. It might be expected, based on the apparent reciprocity of NPR population and circulating NP concentrations in mammals (Anand-Srivastava and Trachte, 1993) that the other species of low salinity acclimated, or FW, fishes examined would decrease plasma NP_{ir} (which they seem to do) and up-regulate NPR-C, a receptor which has been found in teleosts (Donald et al., in press). The present study suggests that in low salinities, the Site 1 receptor population in the hagfish gill is smaller, or decreases its affinity for ANP (Chapter 4). Unfortunately, we do not have the plasma NP_{ir} data to indicate whether there is an increase in circulating NP concentration. Nevertheless, a reduction in the affinity, or the receptor number, of Site 1 receptors would lead to a decrease in cGMP accumulation in the gill, and hence presumably reduce the impact of ANP on Na^+/H^+ exchange, assuming that the antiporter is present in hagfish gill tissue, and that ANP modulates the Na^+/H^+ exchange. The opposite solution may also apply since there is a tendency to increase the sensitivity, or population size, of Site 1 receptors in high salinities (Chapter 4). The present study does not contradict the hypothesis that acid/base regulatory mechanisms in the gill were the precursors of osmoregulatory mechanisms in agnathans invading FW; in fact, future research may well indicate the presence of the Na^+/H^+ antiporter in the hagfish gill, and, furthermore, a modulation of that transport system by NPs. The increased sensitivity of pCNP to NPRs during adjustments to both high and low salinities is unusual and a full interpretation of these results must await additional research (Chapter 4); particularly research that confirms or refutes the presence of an NPR-B type under salinity stress.

Because of structural similarities in the different NP genes, it is presumed that, at least within vertebrates, if not before, gene duplications produced the different members of the NP family: ANP, BNP, and CNP (Rosenzweig and Seidman, 1991). It is assumed that receptor gene duplications also have occurred, as appears to be the case for other peptide hormone receptors, such as those for FSH and LH, andhuman interleukin-8 (Heckert et al., 1992; Lee et al., 1992), because receptor types and subtypes display considerable homologies but are separate gene products (Koller and Goeddel, 1992). By examining the NPR populations in the hagfish, some hypotheses about the timing of those gene duplications can be generated. If the evolution of peptide hormones and receptor partners has taken place by gene duplication, then the following scenarios are possible: (1) either the peptide hormone gene duplicates first, followed by receptor gene duplication, which appears to be the case for the growth hormone and prolactin family (Russell and Nicoll, 1990) and gonadotrophins (Kubokawa, 1990); or (2) the receptor gene may have duplicated initially, which would allow a variation of response to a single peptide in different tissues, assuming there was a differential expression of receptor type in the tissues in question. Examples of (2) appear to be the subtypes of the muscarinic receptors of acetylcholine (Wolfe, 1989), and adenosine receptor subtypes (Collis and Hourani, 1993).

Sequence information on hagfish NPs and their receptors is not at present available. However, it is evident from the fish literature that by the time that bony fish and elasmobranchs diverged, there had been at least one NP gene duplication since both groups contain a CNP gene and bony fish also have an ANP gene (Price et al., 1991; Schofield et al., 1991; Suzuki et al., 1991; Suzuki et al., 1992; Takei et al., 1989). Eels also have a BNP-like gene which has been isolated from eel brains (Takei et al., 1990); however, because this peptide lacks the amino terminal extension typical of BNPs it is difficult to interpret its relationship to other BNPs. Until other fish BNPs have been characterized, it is unknown whether a BNP-like gene is prevalent in fishes. It is evident that at least two, or possibly three, gene duplications occurred in an ancestral gnathostome, or, possibly, an

agnathan ancestor. Given the long phylogenetic history of NPs (Reinecke et al., 1985; Vesely and Giordano, 1992a and b; Turrin et al., 1992; Vesely et al., 1993), it is certainly possible that the gene duplications occurred before the evolution of vertebrates. In terms of NPR evolution, it is clear that both bony fishes and elasmobranchs have a 'promiscuous' type NPR (Gunning et al., 1993; Donald et al., in press); this receptor in teleosts is clearly structurally related to the low molecular weight mammalian NPR-C (Sakaguchi et al., 1993; Donald et al., in press). Both groups also have a GC receptor, in the case of elasmobranchs, it resembles a mammalian NPR-B type (Gunning et al., 1993; Donald, unpublished); the teleost (Gulf toadfish) GC receptor, however, binds ANP and CNP with similar affinity (Donald et al., in press). The presence of both GC-linked and non-GC-linked receptors in both elasmobranchs and bony fishes indicates that the NPR-C type and a GC-linked NPR were present in ancestral gnathostomes. Given the above discussion of only gnathostome fish NPs and receptors, it could be concluded that CNP is the primitive NP, since the other NPs have only been identified in bony fish.

If we include the current knowledge of the NP system in hagfish presented here, what can be further deduced about the evolution of the NP family? The immunological data is suggestive that there is more than one type of NP present in the hagfish; ANP_{ir} has been found in the plasma of Myxine glutinosa (Evans et al., 1989), and also in the heart and the brain (Reinecke et al., 1987). Donald et al. (1992) have also observed BNP-like immunoreactivity in the hagfish brain; however, without NP sequence data it is difficult to draw final conclusions from the use of mammalian antibodies to heterologous NPs. The hagfish receptor data demonstrate a long vertebrate history of NP function in the kidney and the gills. Clearly, the coupling of cell surface NP receptors with particulate guanylate cyclase is phylogenetically ancient, since it is unlikely that this coupling arose separately in hagfish and gnathostome lineages. It is also interesting that, because the GC receptor appears to be an NPR-A type in hagfish (Chapters 2 and 3), ANP, rather than CNP, is indicated as the primitive circulating NP; although, it is also possible that BNP is important

in the hagfish system since the NPR-A receptor in mammals can also bind BNP (Koller and Goeddel, 1992). The apparent elasmobranch condition of having CNP as the major systemic hormone would therefore appear to be a unique characteristic in this group, indicating the presence of at least ANP and CNP before the chondricthyan-bony fish divergence. CNP may also be functional in the hagfish, as ^{125}I-CNP binds in the hagfish brain (Donald and Toop, unpublished), and in this study, there is the intriguing increase in sensitivity of pCNP to ^{125}I-ANP binding sites during salinity stress; one possible explanation for these data is the expression of an NPR-B type receptor. Obviously, further research is needed to clarify this issue. The relationship of the Site 2 'promiscuous' receptor to either the hagfish Site 1 receptor or to NPR-C is impossible to deduce without sequence data; whether this receptor has evolved from an early duplication event which produced the NPR-C in the higher vertebrates, or whether it is the product of a separate event in the hagfish lineage alone, cannot be determined.

The natriuretic peptide family is an ancient peptide hormone family which has been detected in almost all organisms examined, including plants and protists (Reinecke et al., 1985; Vesely and Giordano, 1992a and b; Turrin et al., 1992; Vesely et al., 1993). In fact, certain forms of NPs function in plants in water and solute movement. ProANP $_{(1\text{-}30)}$ proANP $_{(31\text{-}67)}$, but interestingly not ANP $_{(99\text{-}126)}$, increased water movement up stems, transpiration rate, and solute uptake in angiosperms (Vesely, 1993). The present study has not addressed the function of NPs in hagfish. The hypothesis suggested in this study is that the primitive function of NPs was in ion and water regulation, and only later assumed the vasoactive properties observed in vertebrates. Maintenance of a stable internal ionic environment is crucial for successful cellular function in all organisms, and therefore it seems likely that the original role for NPs would be associated with ion regulation, including the regulation of those important in acid/base homeostasis.

Caution is always advisable when interpreting events of vertebrate evolution from a lineage that has been separated from other vertebrate lineages for so long. However, the

current available data on the hagfish NP system strongly suggests that the NP system was already established before the hagfish divergence from the other vertebrates. This history includes duplication of the primitive NP gene, and, minimally, the existence of an ancestral GC-linked, and possibly an ancestral non-GC-linked, receptor. It is necessary to examine the NP system at a phylogenetically earlier point in order to determine the timing of hormone and receptor gene duplications.

LIST OF REFERENCES

Abassi, Z.A., Golomb, E., Klein, H., Keiser, H.R. (1992). Urodilatin: a natriuretic peptide of renal origin. Cardiovascular Drug Revs. 10, 199-210.

Abe, T., Nishiyama, K., Snajdar, R., He, X., Misono, K.S. (1993). Aortic smooth muscle contains guanylate-cyclase-coupled 130-kDa atrial natriuretic factor receptor as predominant receptor form: spontaneous switching to 60-kDa C-receptor upon cell culturing. Eur. J. Biochem. 217, 295-304.

Alt, J.M., Stolte, H., Eisenbach, G.M., Walvig, F. (1981). Renal electrolyte and fluid excretion in the Atlantic hagfish, Myxine glutinosa. J. Exp. Biol. 91, 323-330.

Ando, M., Kondo, K., Takei, Y. (1992). Effects of eel atrial natriuretic peptide on NaCl and water transport across the intestine of the seawater eel. J. Comp. Physiol. 162, 436-439.

Anand-Srivastava, M.B. (1992) Characterization of ANF-R2 receptor-mediated inhibition of adenylate cyclase. Mol. Cell. Biochem. 113, 83-92.

Anand-Srivastava, M.B., Trachte G.J. (1993). Atrial natriuretic factor receptors and signal transduction mechanisms. Pharmacol. Rev. 45, 455-497.

Arnand-Srivastava, M.B., Vinay, P., Genest, J., Cantin, M. (1986). Effect of atrial natriuretic factor on adenylate cyclase in various nephron segments. Am. J. Physiol. 251, F417-F423.

Arnold-Reed, D.E., Balment, R.J. (1991). Atrial natriuretic factor stimulates in-vivo and in-vitro secretion of cortisol in teleosts. J. Endocrinol. 128, R17-R20.

Atlas, S.A., Kleinert, H.D., Camargo, M.J., Januszewicz, A., Sealey, J.E., Laragh, J.H., Schilling, J.W., Lewicki, J.A., Johnson, L.K., Maack, T. (1984). Purification, sequencing, and synthesis of natriuretic and vasoactive rat atrial peptide. Nature. 309, 717-727.

Awazu, M., Ichikawa, I. (1993). Biological significance of atrial natriuretic peptide in the kidney. Nephron. 63, 1-14.

Ballermann, B.J., Hoover, R.L., Karnovsky, M.J., Brenner, B.M. (1985). Physiologic regulation of atrial natriuretic peptide receptors in rat renal glomeruli. J. Clin. Invest. 76, 2049-2056.

Baxter, R.C., Turtle, J.R. (1985). Polypeptide hormone receptors. In The biochemistry of the polypeptide hormones (ed. M. Wallis, S.L. Howell, K.W. Taylor) pp. 410 -433. New York, John Wiley and Sons.

Bellamy, D., Chester Jones, I. (1961). Studies on Myxine glutinosa I. The chemical composition of the tissues. Comp. Biochem. Physiol. 3, 175-183.

Benyajati S., Yokota, S.D. (1990). Renal effects of atrial natriuretic peptide in a marine elasmobranch. Am. J. Physiol. 258, R1201-R1206.

Broadhead, C.L., O'Sullivan, U.T., Deacon, C.F., Henderson, I.W. (1992). Atrial natriuretic peptide in the eel, Anguilla anguilla L.: its cardiac distribution, receptors and actions on isolated branchial cells. J. Molec. Endocrinol. 9, 103-114.

Brenner,B.M., Ballermann, B.J., Gunning, M.E., Zeidel M.L. (1990). Diverse biological actions of atrial natriuretic peptide. Physiol. Rev. 70(3), 665-699.

Brown, J., Zuo, Z. (1992). Renal receptors for atrial and C-type natriuretic peptides in the rat. Am. J. Physiol. 263, F89-F96.

Cahill, P.A., Redmond, E.M., Keenan, A.K. (1990). Vascular atrial natriuretic factor receptor subtypes are not independently regulated by atrial peptides. J. Biol. Chem. 265 (35), 21896-21906.

Cameron, J.N. (1989). The respiratory physiology of animals. 353 pp. New York, Oxford, Oxford University Press.

Canaan-Kuhl, S., Jamison, R.L., Myers, B.D., Pratt, R.E. (1992). Identification of "B" receptor for natriuretic peptide in human kidney. Endocrinology. 130, 550-552.

Caramelo, C., Lopez-Farre, A., Riesco, A., Olivera, A., Okada, K., Cragoe, E.J. Jr., Tsai, P., Briner, V.A., Schrier, R.W. (1994). Atrial natriuretic peptide and cGMP inhibit Na^+/H^+antiporter in vascular smooth muscle cells in culture. Kid. Int. 45, 66-75.

Cerra, M.C., Canonaco, M., Tota, B. (1992). A quantitative autoradiographical study of 125I atrial natriuretic factor in the heart of a teleost fish (Conger conger). J. Exp. Zool. 263, 215-219.

Chinkers, M., Garbers, D.L. (1991). Signal transduction by guanylyl cyclases. Ann. Rev. Biochem. 60, 553-575.

Cholette, C., Gagnon, A., Germain, P. (1970). Isosmotic adaptation in Myxine glutinosa L. I. Variations of some parameters and role of the amino acid pool of the muscle cells. Comp. Biochem. Physiol. 33, 333-346.

Collis, M.G., Hourani, S.M. (1993). Adenosine receptor subtypes. TiPS. 14, 360-366.

Conley, D.M., Mallatt, J. (1988). Histochemical localization of Na^+-K^+ ATPase and carbonic anhydrase activity in gills of 17 fish species. Can. J. Zoo. 66, 2398-2405.

Currie, M.G., Geller, D.M., Cole, B.R., Siegel, N.R., Fok, K.F., Adams, S.P., Eubanks, S.R., Galluppi, G.R., Needleman, P. (1984). Purification and sequence analysis of bioactive atrial peptides (atriopeptins). Science. 223, 67-69.

de Bold, A.J., Borenstein, H.B., Veress, A.T. Sonnenberg, H. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. Life Sci. 28, 89-94.

de Bold, A.J., Raymond, J., Bencosme, S.A. (1978). Atrial specific granules of the rat heart: light microscopic staining and histochemical reactions. J. Histochem. Cytochem. 26, 1094-1102.

Donald, J.A., Evans D.H. (1992). Immunohistochemical localisation of natriuretic peptides in the heart and brain of the gulf toadfish, Opsanus beta. Cell Tiss. Res. 269, 151-158.

Donald, J.A., Toop, T., Evans, D.H. (1994). Localization and analysis of natriuretic peptide receptors in the gills of the gulf toadfish, Opsanus beta (Teleostei). Am. J. Physiol. In press.

Donald, J.A, Vomachka, A.J., Evans D.H. (1992). Immunohistochemical localisation of natriuretic peptides in the brains and hearts of the spiny dogfish Squalus acanthias and the Atlantic hagfish Myxine glutinosa. Cell Tissue Res. 270, 535-545.

Dousa, T.P., Shah, S.V., Abboud, H.E. (1980). Potential role of cyclic nucleotides in glomerular pathophysiology. In Advances in cyclic nucleotide research. Vol. 12 (ed. P. Hamet and H. Sands) pp. 285-299. New York, Raven Press.

Duff, D.W., Olson, K.R. (1986). Trout vascular and renal responses to atrial natriuretic factor and heart extracts. Am. J. Physiol. 251, R639-R642.

Duff, D.W., Olson, K.R. (1992). Atrial natriuretic peptide clearance receptors in trout: effects of receptor inhibition in vivo. J. Exp. Zool. 262, 343-346.

Elger, M. (1987). The branchial circulation and gill epithelia in the Atlantic hagfish, Myxine glutinosa L. Anat. Embryol. 175, 489-504.

Evans, D.H. (1984). Gill Na+/H+ and Cl-/HCO3- exchange systems evolved before the vertebrates entered fresh water. J. Exp. Biol. 113, 465-469.

Evans, D.H. (1990). An emerging role for a cardiac peptide hormone in fish osmoregulation. Ann. Rev. Physiol. 52, 43-60.

Evans, D.H. (1991). Rat atriopeptin dilates vascular smooth muscle of the ventral aorta from the shark (Squalus acanthias) and the hagfish (Myxine glutinosa). J. exp. Biol. 157, 551-555.

Evans, D.H. (1993). Osmotic and ionic regulation. In The physiology of fishes (ed. D.H. Evans), pp. 315-341. Boca Raton: CRC Press.

Evans D.H. (1994). The roles of natriuretic peptide hormones (NPs) in fish osmoregulation and hemodynamics. In Advances in Environmental and comparative physiology--mechanisms of systemic regulation in lower vertebrates: II: acid-base regulation, ion transfer and metabolism (ed. N. Heisler), Heidelberg: Springer-Verlag. In press.

Evans, D.H., Takei,Y. (1992). A putative role for natriuretic peptides in fish osmoregulation. NIPS. 7, 15-19.

Evans, D.H., Donald, J.A., Stidham, J.D. (1993) C-Type natriuretic peptides are not particularly potent dilators of hagfish (Myxine glutinosa) vascular smooth muscle. Bull. Mt. Desert Is. Biol. Lab. 32, 106.

Evans, D.H., Toop, T., Donald, J., Forrest, J.N. Jr. (1992). C-type natriuretic peptides are potent dilators of shark vascular smooth muscle. J. Exp. Zool. 264, 84-87.

Evans, D.H., Chipouras, E., Payne, J.A. (1989). Immunoreactive atriopeptin in plasma of fishes: its potential role in gill hemodynamics. Am. J. Physiol. 257, R939-R945.

Fels, L.M., Raguse-Degener, G., Stolte, H. (1989). The archinephron of Myxine glutinosa L. (Cyclostomata). In Structure and function of the kidney (ed. R.K.H. Kinne), Comp. Physiol. 1, pp. 73-102. Basel, Karger.

Féthière, J., Graihle, R., De Léan, A. (1992). Identification of the atrial natriuretic factor-R_{ic} receptor subtype (B clone) in cultured rat aortic smooth muscle cells. FEBS Letts. 305, 77-80.

Forey, P., Janvier, P. (1993). Agnathans and the origin of jawed vertebrates. Nature. 361, 129-134.

Forrest, J.N. Jr, Kelley, G.G., Opdyke, D., Schofield, J.P., Aller, C. (1992). Synthetic shark CNP based on the amino acid sequence of pre-pro shark heart CNP potentially stimulates chloride secretion in the perfused shark rectal gland. Bull. Mt. Desert Isl. Biol. Lab. 31, 71-72.

Freeman, J.D., Bernard, R.A. (1990). Atrial natriuretic peptide and salt adaptation in the sea lamprey, Petromyzon marinus. The Physiologist. 33, A-38.

Gauquelin, G., Garcia, R., Carrier, F., Cantin, M., Gutkowska, J., Thibault, G., Schiffrin, E.L. (1988). Glomerular ANF receptor regulation during changes in sodium and water metabolism. Am. J. Physiol. 254, F51-F55.

Genest, G., Cantin, M. (1988). The atrial natriuretic factor: its physiology and biochemistry. Rev. Physiol. Biochem. Pharmacol. 110, 2-145.

Gilbert, C.R. (1993). Evolution and Phylogeny. In The physiology of fishes (ed. D.H. Evans) pp.1-45. Boca Raton, CRC Press.

Goetz, K.L. (1991). Renal natriuretic peptide (urodilatin?) and atriopeptin: evolving concepts. Am. J. Physiol. 261, F921-F932.

Goetz, K.L. (1993). Is urodilatin (rather than atriopeptin) the primary natriuretic peptide of the ANP family? J. Cardiovascular Pharmacol. 22(Suppl. 2) S84-S85.

Greenwald, J.E., Ritter, D., Tetens, E., Rotwein, R.S. (1992). Renal expression of the gene for atrial natriuretic factor. Am. J. Physiol. 263, F974-F978.

Griffith, R.W. (1985). Habitat, phylogeny, and the evolution of osmoregulatory strategies in primitive fishes. In Evolutionary biology of primitive fishes (ed. R.E. Foreman, A. Gorbman, J.M. Dodd, R. Olsson) pp. 69-80. New York and London, NATO ASI Series, Plenum Press.

Gunning, M., Cuero, C., Solomon, R., Silva P. (1993). C-type natriuretic peptide receptors and signaling in rectal gland of Squalus acanthias. Am. J. Physiol. 264, F300-F305.

Gunning, M., Silva, P., Brenner, B.M., Zeidel, M.L. (1989). Characteristics of ANP-sensitive guanylate cyclase in inner medullary collecting duct cells. Am. J. Physiol. 256, F766-F775.

Halstead, L.B. (1985). The vertebrate invasion of fresh water. Phil. Trans. R. Soc. Lond. B 309, 243-258.

Hardisty, M.W. (1979). Biology of the Cyclostomes. London, Chapman and Hall. 429 pp.

Heckert, L.L., Daley, I.J., Griswold, M.D. (1992). Structural organization of the follicle-stimulating hormone receptor gene. Mol. Endocrinol. 6, 70-80.

Hentschel, H., Elger, M. (1989). Morphology of glomerular and aglomerular kidneys. In Structure and function of the kidney (ed. R.K.H. Kinne) pp. 1-72. Comparative Physiology, Basel, Karger.

Hosoda, K., Nakao, K., Mukoyama, M., Saito, Y., Jougasaki, M., Shirakami, G., Suga, S., Ogawa, Y., Yasue, H., Imura, H. (1991). Expression of brain natriuretic peptide in gene in human heart: production in the ventricle. Hypertension. 17, 1152-1155.

Hubbard, J.R. (1987). Growth hormone receptors. In Peptide hormone receptors (ed. M.Y. Kalimi, J.R. Hubbard) pp. 1 - 61. Berlin, New York, Walter de Gruyter.

Karnaky K.J. Jr., Stidham, J.D., Nelson, D.S., McCraw, A.S., Valentich, J.D., Kennedy, M.P., Currie, M.G. (1992). C-type natriuretic peptide is a potent secretagogue for the cultured shark (Squalus acanthias) rectal gland. Bull. Mt. Desert. Isl. Biol. Lab. 31, 122-123.

Karnaky, K.J. Jr, Valentich, J.D., Currie, M.G., Oehlenschlager, W.F., Kennedy, M.P. (1991). Atriopeptin stimulates chloride secretion in cultured shark rectal gland cells. Am. J. Physiol. 260, C1125-C1130.

Katafuchi, T., Mizuno, T., Hagiwara, H., Itakura, M., Ito, T., Hirose, S. (1992). Modulation by NaCl of atrial natriuretic peptide receptor levels and cyclic GMP responsiveness to atrial natriuretic peptide of cultured vascular endothelial cells. J. Biol. Chem. 267, 7624-7629.

Kato, J., Lanier-Smith, K.L., Currie, M.G. (1991). Cyclic GMP down-regulates atrial natriuretic peptide receptors on cultured vascular endothelial cells. J. Biol. Chem. 266, 14681-14685.

Kirschner, L.B. (1979). Control mechanisms in crustaceans and fishes. In Mechanisms of osmoregulation in animals (ed. R. Gilles) pp. 157-222. New York, Wiley-Interscience.

Kisch, B. (1956). Electron microscopy of the atrium of the heart: I. The guinea pig. Exp. Med. Surg. 14, 99-112.

Kloas, W., Flugge, G., Fuchs, E., Stolte, H. (1988). Binding sites for atrial natriuretic peptide in the kidney and aorta of the hagfish (Myxine glutinosa). Comp. Biochem. Physiol. 91A, 685-688.

Kollenda, M.C., Vollmar, A.M., McEnroe, G.A., Gerbes, A.L. (1990). Dehydration increases the density of C receptors for ANF on rat glomerular membranes. Am. J. Physiol. 258, R1084-R1088.

Koller, K.J., Goeddel, D.V. (1992). Molecular biology of the natriuretic peptides and their receptors. Circulation 86, 1081-1088.

Konrad, E.M., Thibault, G., Schiffrin, E.L. (1992). Autoradiographic visualization of the natriuretic peptide receptor-B in rat tissues. Regulatory Peptides. 39, 177-189.

Kubokawa, K. (1990). Evolution and adaptation of gonadotropin receptors. In Progress in comparative endocrinology (ed. A. Epple, C.G. Scanes, M.H. Stetson) pp. 157-162. New York, Wiley-Liss.

Lee, J., Horuk, R., Rice, G.C., Bennett, G.L., Camerato, T., Wood, W.I. (1992). Characterization of two high affinity human interleukin-8 receptors. J. Biol. Chem. 267, 16283-16287.

Lee, J., Malvin, R.L. (1987). Natriuretic response to homologous heart extract in aglomerular toadfish. Am. J. Physiol. 252, R1055-R1058.

Leitman, D.C., Murad, F. (1990). Structure and function of atrial natriuretic peptide subtypes. In Atrial natriuretic peptides (ed. W.K. Samson and R. Quirion), pp. 78-93. Boca Raton: CRC Press.

Levin, E.R. (1993). Natriuretic peptide C-receptor: more than a clearance receptor. Am. J. Physiol. 264, E483-E489.

McFarland, W.N., Munz, F.W. (1958). A re-examination of the osmotic properties of the Pacific hagfish, Polistotrema stouti. Biol. Bull. Woods Hole. 114, 348-356.

McFarland, W.N., Munz, F.W. (1965). Regulation of body weight and serum composition by hagfish in various media. Comp. Biochem. Physiol. 14, 383-398.

McPherson, G. (1985). Kinetic EBDA, Ligand, Lowry. A collection of radioligand binding analysis programs. Amsterdam: Elsevier. 126pp.

Maack, T. (1992). Receptors of atrial natriuretic factor. Ann. Rev. Physiol. 54, 11-27.

Maack, T., Suzuki, M., Almeida, F.A., Nussenzweig, D., Scarborough, R.M., McEnroe, G.A., Lewicki, J.A. (1987). Physiological role of silent receptors of atrial natriuretic factor. Science. 238, 675-678.

Mallatt, J. (1984). Early vertebrate evolution: pharyngeal structure and the origin of gnathostomes. J. Zool. Lond. 204, 169-183.

Mallatt, J., Paulsen, C. (1986). Gill ultrastructure of the Pacific hagfish Eptatretus stouti. Am. J. Anat. 177, 243-269.

Mallatt, J., Conley, D.M., Ridgway, R.L. (1987). Why do hagfish have gill "chloride cells" when they need not regulate plasma NaCl concentration? Can. J. Zool. 65, 1956-1965.

Martin, E.R, Lewicki, J.A., Scarborough, R.M., Ballerman, B.J. (1989). Expression and regulation of ANP receptor subtypes in rat renal glomeruli and papillae. Am . J. Physiol. 257, F649-F657.

Morris, R. (1960). General problems of osmoregulation with special reference to cyclostomes. Symp. Zool. Soc. Lond. 1, 1-15.

Munz, F.W., McFarland, W.N. (1964). Regulatory function of a primitive vertebrate kidney. Comp. Biochem. Physiol. 13, 281-400.

Nakao, K., Itoh, H., Kambashayashi, Y., Hosoda, K., Saito, Y., Yamada, T., Mukoyama, M., Arai, H., Shirakami, G., Suga, S., Jougasaki, M., Ogawa, Y., Inouye, K., Imura, H. (1990). Rat brain natriuretic peptide. Isolation from rat heart and tissue distribution. Hypertension. 15, 774-778.

O' Grady, S.M., Field, N., Nash, N.T., Rao, M.C. (1985). Atrial natriuretic factor inhibits Na-K-Cl cotransport in teleost intestine. Am. J. Physiol. 249, C531-C534.

Oglesby, J. (1981). Volume regulation in aquatic invertebrates. J. Exp. Zool. 215, 289-301.

Olson, K.R., Duff, D.W. (1992). Cardiovascular and renal effects of eel and rat atrial natriuretic peptide in rainbow trout, Salmo gairdneri. J. Comp. Physiol. 162, 408-415.

Olson, K.R., Duff, D.W. (1993). Single-pass gill extraction and tissue distribution of atrial natriuretic peptide in trout. Am. J. Physiol. 265, R124-R131.

Olson, K.R., Meisheri, K.D. (1989). Effects of atrial natriuretic factor on isolated arteries and perfused organs of trout. Am. J. Physiol. 256, R10-R18.

Perrott, M.N., Sainsbury, R.J. and Balment, R.J. (1993). Peptide hormone-stimulated second messenger production in the teleosten nephron. Gen. and Comp. Endocrin. 89, 387-395.

Perry, S.F., Laurent, P. (1989). Adaptational responses of rainbow trout to lowered external NaCl concentration: contribution of the chloride cell. J. Exp. Biol. 147, 147-168.

Price, D.A., Doble, K.E., Lee, T.D., Galli, S.M., Dunn, B.M., Parten, B., Evans, D.H. (1991). The sequencing, synthesis and biological actions of an ANP-like peptide isolated from the brain of the killifish, Fundulus heteroclitus. Biol. Bull. Woods Hole. 178, 279-285.

Redmond, E.M., Cahill, P.A., Keenan, A.K. (1990). Atrial natriuretic factor recognizes two receptor subtypes in endothelial cells cultured from bovine pulmonary artery. FEBS Letts. 269, 157-162.

Reinecke, M., Betzler, D., Forssmann, W.G. (1987). Immunocytochemistry of cardiac polypeptide hormones (cardiodilatin/atrial natriuretic polypeptide) in brain and hearts of Myxine glutinosa (Cyclostomata). Histochemistry. 86, 233-239.

Reinecke, M., Nehls, M., Forssmann, W.G. (1985). Phylogenetic aspects of cardiac hormones as revealed by immunocytochemistry, electronmicroscopy, and bioassay. Peptides. 6 (Suppl. 3), 321-331.

Robertson, J.D. (1957). The habitat of the early vertebrates. Biol. Rev. 32, 156-187.

Robertson, J.D. (1963). Osmoregulation and ionic composition of cells and tissues. In The biology of Myxine (ed. A. Brodal, R. Fänge) pp. 504-515. Norway, Universitetsforlaget.

Robertson, J.D. (1966). Osmotic constituents of the blood plasma and parietal muscle of Myxine glutinosa L. In Some contemporary studies in marine science (ed. H. Barnes) pp. 631-644. London, Allen and Unwin.

Romer, A.S., Grove, B.H. (1935). Environment of the early vertebrates. Amer. Midl. Nat. 16, 805-856.

Rosensweig, A., Seidman, C.E. (1991). Atrial natriuretic factor and related peptide hormones. Annu. Rev. Biochem. 60, 229-255.

Russell, S.M., Nicoll, C.S. (1990). Evolution of growth hormone and prolactin receptors and effectors. In Progress in comparative endocrinology (ed. A. Epple, C.G. Scanes, M.H. Stetson) pp. 157-162. New York, Wiley-Liss.

Ruskoaho, H. (1992). Atrial natriuretic peptide: synthesis, release, and metabolism. Physiol. Rev. 44(4), 479-602.

Sakaguchi, H., Katafuchi, T., Hagiwara, H., Takei, Y. and Hirose, S. (1993). High-density localization of ANP receptors in chondrocytes of eel gill cartilage. Am. J. Physiol. 265, R474-R479.

Samson, W.K. (1990). Neuroendocrine actions of the atrial natriuretic peptides. In Atrial natriuretic peptides (ed.W.K. Samson and R. Quirion) pp. 231-241. Boca Raton: CRC Press.

Samson WK, Quirion R (ed.) (1990) Atrial natriuretic peptides. Boca Raton, CRC Press. 304 pp.

Schiede, J.I., Zadunaisky, J.A. (1988). Effect of atriopeptin II on isolated opercular epithelium of Fundulus heteroclitus. Am. J. Physiol. 254, R27-R32.

Schofield, J.P., Jones, D.S.C., Forrest, J.N. (1991). Identification of C-type natriuretic peptide in heart of spiny dogfish shark (Squalus acanthias). Am. J. Physiol. 261, F734-F739.

Schulz-Knappe, P., Forssmann, K., Herbst, F., Hock, D., Pipkorn., Forssmann, W.G. (1988). Isolation and structural analysis of urodilatin, a new peptide of the cardiodilatin-(ANP)-family, extracted from human urine. Klin. Wochenschr. 66, 752-759.

Semrad, C.E., Cragoe, E.J. Jr., Chang, E.B. (1990). Inhibition of Na/H exchange in avian intestine by atrial natriuretic factor. J. Clin. Invest. 86, 585-591.

Smith, Homer W. (1932). Water regulation and its evolution in the fishes. Quart. Rev. Biol. 7, 1-26.

Smith N.F., Eddy, F.B., Struthers, A.D., Talbot, C. (1991). Renin, atrial natriuretic peptide and blood plasma ions in parr and smolts of Atlantic salmon Salmo salar L. and rainbow trout Oncorhynchus mykiss (Walbaum) in fresh water and after short-term exposure to sea water. J. Exp. Biol. 157, 63-74.

Solomon, R., Protter, A., McEnroe, G., Porter, J.G., Silva, P. (1992). C-type natriuretic peptides stimulate chloride secretion in the rectal gland of Squalus acanthias. Am. J. Physiol. 262, R707-R711.

Solomon, R., Taylor, M., Dorsey, D., Silva, P., Epstein, F.H. (1985). Atriopeptin stimulation of rectal gland in Squalus acanthius. Am. J. Physiol. 249, R348-R354.

Sonnenberg, H. (1990). Mechanisms of release and renal action of atrial natriuretic factor. Acta Physiol. Scand. 139 (Suppl. 591), 80-87.

Stingo, A.J., Clavell, A.L., Aarhus, L.L., Brunett, J.C. Jr. (1992). Cardiovascular and renal actions of C-type natriuretic peptide. Am. J. Physiol. 262, H308-H312.

Sudoh, T., Kangawa, K., Minamino, N., Matsuo, H. (1988). A new natriuretic peptide in porcine brain. Nature. 332, 78-81.

Sudoh, T., Minamino, N., Kangawa, N., Matsuo, H. (1990). C-type natriuretic peptide (CNP): a new member of natriuretic family identified in porcine brain. Biochem. Biophys. Res. Commun. 168, 863-870.

Suga, S., Kazuwa, N., Kiminori, H., Mukoyama, M., Ogawa, Y., Shirakami, G., Arai, H., Saito, Y., Kambayashi, Y., Inouye, K., Imura, H (1992a). Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology. 130, 229-239.

Suga, S., Nakao, K., Mukoyama, M., Arai, H., Hosoda, K., Ogawa, Y., Imura, H. (1992b). Characterization of natriuretic peptide receptors in cultured cells. Hypertension. 19, 762-765.

Suzuki, R., Takahashi, A., Hazon, N., Takei, Y. (1991). Isolation of high-molecular-weight C-type natriuretic peptide from the heart of a cartilaginous fish (European dogfish, Scyliorhinus canicula). FEBS Lett. 282, 321-325.

Suzuki, R., Takahashi, A., Takei, Y. (1992). Different molecular forms of C-type natriuretic peptide isolated from the brain and heart of an elasmobranch, Triakis scyllia. J. Endocrinol. 135, 317-323.

Takei, Y., Balment, R.J. (1993). Biochemistry and physiology of eel natriuretic peptides. Fish Physiol. Biochem. 11, 183-188.

Takei, Y., Takahashi, A., Watanabe, T.X., Nakajima, K., Sakakibara, S. (1989). Amino acid sequence and relative biological activity of eel atrial natriuretic peptide. Biochem. Biophys. Res. Comm. 164, 537-543.

Takei, Y., Takahashi, A., Watanabe, T.X., Nakajima, K. and Sakakibara, S., Takao, T. and Shimonishi, Y. (1990). Amino acid sequence and relative biological activity of a natriuretic peptide isolated from eel brain. Biochem. Biophys. Res. Comm. 170, 883-891.

Takei, Y., Takahashi, A., Watanabe, T.X., Nakajima, K.S., Sahakibara S. (1991). A novel natriuretic peptide isolated from eel cardiac ventricles. FEBS Lett. 282, 317-320.

Terada Y., Moriyama, T., Martin, B.M., Knepper, M.K., Garcia-Perez, A. (1991). RT-PCR microlocalization of mRNA for guanylyl cyclase-coupled ANF receptor in rat kidney. Am. J. Physiol. 261, F1080-F1087.

Turrin, M.Q.A., Sawaya, M.I., Santos, M.C.F., Veiga, L.V., Mantero, F., Opocher, G. (1992). Atrial natriuretic peptide (ANP) increases in the mangrove crab Ucides cordatus when exposed to increased environmental salinity. Comp. Biochem. Physiol. 101A, 803-806.

Uemura, H., Naruse, M., Hirohama, T., Nakamura, S., Kasuya, Y., Aoto, T. (1990). Immunoreactive atrial natriuretic peptide in the fish heart and blood plasma examined by electron microscopy, immunohistochemistry and radioimmunoassay. Cell Tissue Res. 260, 235-247.

Uva, M.B., Masini, M.A., Napoli, L., Devecchi, M. (1993). Immunoreactive atrial natriuretic-like peptide in antarctic teleosts. Comp. Biochem. Physiol. 104A, 291-297.

Valentin, J-P., Sechi, L.A. Qiu, C., Schambelan, M., Humphreys, M.H. (1993). Urodilatin binds to and activates renal receptors for atrial natriuretic peptide. Hypertension. 21, 432-438.

Vallarino, M., Feuilloley, M., Gutkowska, J., Cantin, M. and Vaudry, H. (1990). Localisation of atrial natriuretic factor (ANF)-related peptides in the central nervous system of the elasmobranch fish Scyliorhinus canicula. Peptides. 11, 1175-1181.

Vesely, D.L., Giordano, A.T. (1992a). Atrial natriuretic factor-like peptide and its prohormone within single cell orgainisms. Peptides 13, 177-182.

Vesely, D.L., Giordano, A.T. (1992b). The most primitive heart in the animal kingdom contains the atrial natriuretic peptide hormonal system. Comp. Biochem. Physiol. 101C, 325-329.

Vesely, D.L., Gower, W.R. Jr., Giordano, A.T. (1993). Atrial natriuretic peptides are present throughout the plant kingdom and enhance solute flow in plants. Am. J. Physiol. 265, E465-E477.

Wendelaar Bonga (1993). Endocrinology. In The physiology of fishes (ed. D.H. Evans) pp. 469-502. Boca Raton: CRC Press.

Westenfelder C., Birch, F.M., Baranowski R.L., Rosenfeld, M.J., Shiozawa, D.K., Kablitz, C. (1988). Atrial natriuretic factor and salt adaptation in the teleost fish Gila atraria. Am. J. Physiol. 255, F1281-F1286.

Wolfe, B.B. (1989). Subtypes of muscarinic cholinergic receptors. In The muscarinic receptors (ed. J.H. Brown) pp.125-150. Clifton, New Jersey, Humana Press.

Zeidel, M.L., Brenner, B.M. (1987). Actions of atrial natriuretic peptides on the kidney. Seminars in Nephrology. 7, 91-97.

BIOGRAPHICAL SKETCH

Marie-Thérèse Toop was born in Bridgwater, Somerset, England. She grew up in rural Somerset, attending the Holy Rosary nursery school, followed by ten years, from 1961-1971, as a boarder at La Retraite convent, Burnham-on-Sea, Somerset, during which time she made acquaintance with English literature and estuarine organisms. On completion of her high school education, she spent three years in various employments, including a year (1972) as an English nanny in Geneva, Switzerland. From 1974 to 1979 she was registered at the University of Birmingham, England, where she completed her Bachelor of Arts degree in English language and literature (1977), and subsequently her Master of Arts degree in Victorian literature (1979). The title of her master's thesis is A Study of 'Aylwin' by Theodore Watts-Dunton. In 1979 she moved to Gainesville, Florida. From 1983 to 1987 she was an undergraduate at the University of Florida, Gainesville. She completed her Bachelor of Science degree in zoology in 1987, and since that time has been a graduate student in the Department of Zoology. In 1987 she began her master's research, completing her thesis entitled Some behavioral and physiological responses of the ascidian, *Styela plicata* (Lesueur, 1823), during acclimation to low salinity in 1990. Following the completion of her doctorate she will return as a postdoctoral fellow to the laboratory of her supervisor, Dr. David H. Evans, where she will continue to work on the evolution of natriuretic peptide systems in fishes.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David H. Evans, Chairman
Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Louis J. Guillette Jr.
Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michele G. Wheatly
Associate Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Debopam Chakrabarti
Assistant Professor of Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael M. Miyamoto
Associate Professor of Zoology

This dissertation was submitted to the Graduate Faculty of the Department of Zoology in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1994

Dean, Graduate School